Vol. 55, 1976

Acta Haematologica

International Journal of Haematology
Journal International d'Hématologie
Internationale Zeitschrift für Hämatologie

Official Organ of the European Division of the International Society of Haematology

S. Karger · Basel · München · Paris · London · New York · Sydney

S. Karger · Basel · München · Paris · London · New York · Sydney
Arnold-Böcklin-Strasse 25, CH–4011 Basel (Switzerland)

Printed in Switzerland by National-Zeitung AG, Basel

Index

No 1

No 2

No 3

No 4

No 5

No. 6

Indexes

Acta haemat 55 1–9 (1976)

Monocyte Ingestion of IgG-Coated Erythrocytes from Haemolysing and Non-Haemolysing Subjects

D Y MASON

Department of Pathology, Gibson Laboratories, Radcliffe Infirmary, Oxford

Key Words Antibodies Autoimmune haemolysis Erythrocyte phagocytosis Haemolytic anaemia Monocytes

Abstract A number of individuals in the population, including healthy blood donors and patients on methyldopa, show evidence of erythrocyte coating by IgG autoantibodies (positive direct Coombs test) without signs of a haemolytic state The readiness with which red cells from these subjects are taken up by normal human monocytes in short term culture was examined and compared with the phagocytosis of red cells from cases of IgG mediated haemolytic anaemia Red cells from non haemolysing subjects were as avidly ingested in this system as were red cells from haemolysing patients Similar results were obtained using normal red cells sensitised with autoantibody eluted from the cells of a healthy blood donor

The majority of IgG erythrocyte antibodies are unable to initiate complement activation They can only cause haemolysis via the agency of mononuclear phagocytic cells These cells, which are localised predominantly in the liver and spleen, carry membrane receptors for the Fc region of IgG enabling them to bind and subsequently engulf antibody-coated red cells [1, 2] Apparently identical Fc receptors are carried on circulating blood monocytes, the immediate precursors of tissue phagocytes The demonstration of this functional similarity between monocytes and tissue histiocytes has led to proposals that the *in vitro* interaction between isolated blood monocytes and antibody-sensitised red cells provides a valuable model for the mechanism of *in vivo* haemolysis [1, 3, 4]

Antibody-coated red cells are not always preferentially removed from the circulation, however, and a strongly positive direct Coombs' test (DCT) is compatible with normal red cell survival Such a situation is encountered relatively frequently in hypertensive patients treated with meth-

yldopa [5], it may also occur, for no identifiable reason in healthy subjects The frequency of this phenomenon in a donor population has been estimated at between 1 and 3 per 10,000 [6], and in at least one such subject red cell survival has been directly studied by ^{51}Cr labelling and shown to be normal [7]

The present study was designed to compare the avidity with which DCT-positive red cells from actively haemolysing and non haemolysing subjects are engulfed by normal blood monocytes If red cells from the latter group are less readily phagocytosed it would suggest a difference in the type, quantity or configuration of erythrocyte-bound IgG between the two groups and thus explain the two different clinical pictures Equally active phagocytosis of red cells from each group, on the other hand, would imply either that this *in vitro* model is not a valid analogue of haemolysis *in vivo*, or alternatively that other characteristics, peculiar to the subjects themselves, distinguish the haemolysing from the non-haemolysing DCT positive individual

Subjects, Materials and Methods

A total of 23 DCT positive subjects were studied They fell into four categories

Cases of warm autoimmune haemolytic anaemia [11] All of these patients were diagnosed by standard haematological criteria and manifested strongly positive direct Coombs tests with anti IgG reagents All but one of these patients were actively haemolysing when studied The exception had been in haematological remission and receiving no therapy for several years and is included in the non haemolysing category (fig 2)

Babies suffering from Rhesus haemolytic disease [3] Each of these showed evidence of accelerated haemolysis at the time of testing

Hypertensive patients on methyldopa [6] Methyldopa treated patients were screened regularly for evidence of red cell autoantibody formation and samples were obtained from those with strongly positive IgG direct Coombs tests

Blood donors [3] These subjects were found to have positive IgG direct Coombs tests in the course of routine serological testing Each of them were healthy and haematologically normal

Red cell samples Blood samples were stored as clotted or EDTA or ACD anti coagulated specimens for up to 5 days at 4 °C before testing Preliminary studies showed that samples stored in this way did not alter significantly in reactivity in the phagocytosis system Immediately before assay red cells were washed 3 times in normal saline and suspended at a haematocrit of 5% in Hanks solution containing 10% fetal calf serum

IgG sensitised red cell controls Aliquots of heparin anti coagulated red cells from the blood samples which provided normal monocytes for the same experiment

(see below) were washed 3 times in normal saline and incubated at 37 °C with equal volumes of dilutions of a standard Rhesus anti D antiserum This reagent comprised a pool of sera from four maternally iso immunised D negative women After 30 min incubation the red cells were again washed 3 times in normal saline and suspended in Hanks solution containing 10% fetal calf serum

Monocyte phagocytosis assay This technique is essentially identical to the method described by ABRAMSON *et al* [8] For each experiment venous blood was obtained from the same healthy normal subject and mixed with a half volume of 3% dextran in normal saline containing 10 units/ml of preservative free heparin After 1–1 5 h sedimentation at room temperature the white cells in the supernatant were washed 3 times in Hanks solution and suspended at a concentration of 6–8×10⁶/ml in Hanks solution containing 10% fetal calf serum Aliquots of the cell suspension (0 5 ml) were dispensed into 16 mm diameter flat bottomed plastic wells (Linbro Chemical Co Code FB54) After 1 h at room temperature during which time cells sedimented to the floors of the wells non adherent cells were removed by washing with two changes of Hanks solution Preliminary experiments established that the great majority of cells remaining after this step were monocytes and polymorphs The wells were then filled with 0.5 ml of red cell suspension each red cell sample being tested in duplicate The wells were sealed with parafilm and incubated at 37 °C for 2 75 h Non adherent red cells were removed by a single wash with Hanks solution followed by two washes in isotonic pH 7 4 buffered saline containing 2% bovine serum albumin The wells were air dried, fixed for 5 min in methanol and Giemsa stained The number of red cells engulfed by 200 monocytes was counted for each well and the mean of each duplicate calculated

Calculation of strength of opsonisation Red cells sensitised with IgG were avidly phagocytosed by monocytes under the conditions of these experiments and the degree of phagocytosis was directly related to the concentration of sensitising antiserum This relationship is illustrated in figure 1

Red cell samples from DCT positive subjects were tested in batches of between one and four at a time in the phagocytosis assay It was necessary to compensate for possible between experiment variations in the activity of monocyte monolayers prepared on different occasions This was achieved by including two IgG sensitised red cell controls in each experiment These controls consisted of red cells sensitised with two dilutions of the standard antiserum (0 125 and 0 03125) chosen because they had been shown to cause strong and weak phagocytosis, respectively (fig 1) The degree of phagocytosis of these two standards in any experiment was plotted on a log scale against the two serum dilutions, and a straight line drawn through these two points (giving a slope similar to that illustrated in figure 1) This graph was then used to convert phagocytosis figures for each DCT positive red cell sample tested in the same experiment into strength of opsonisation This value is the concentration of the standard antiserum which would have caused the same degree of phagocytosis if it were used to sensitise normal red cells for testing against the same monocyte monolayer

Elution of antibody from direct Coombs positive red cells The acid elution technique described by KIDD [9] was used for this purpose The red cells had been stored in ACD at 4 °C before elution

yldopa [5], it may also occur, for no identifiable reason in healthy subjects The frequency of this phenomenon in a donor population has been estimated at between 1 and 3 per 10,000 [6], and in at least one such subject red cell survival has been directly studied by ^{51}Cr labelling and shown to be normal [7]

The present study was designed to compare the avidity with which DCT-positive red cells from actively haemolysing and non haemolysing subjects are engulfed by normal blood monocytes If red cells from the latter group are less readily phagocytosed it would suggest a difference in the type, quantity or configuration of erythrocyte bound IgG between the two groups and thus explain the two different clinical pictures Equally active phagocytosis of red cells from each group, on the other hand, would imply either that this *in vitro* model is not a valid analogue of haemolysis *in vivo*, or alternatively that other characteristics peculiar to the subjects themselves, distinguish the haemolysing from the non haemolysing DCT-positive individual

Subjects Materials and Methods

A total of 23 DCT positive subjects were studied They fell into four categories

Cases of warm autoimmune haemolytic anaemia [11] All of these patients were diagnosed by standard haematological criteria and manifested strongly positive direct Coombs tests with anti IgG reagents All but one of these patients were actively haemolysing when studied The exception had been in haematological remission and receiving no therapy for several years and is included in the non haemolysing category (fig 2)

Babies suffering from Rhesus haemolytic disease [3] Each of these showed evidence of accelerated haemolysis at the time of testing

Hypertensive patients on methyldopa [6] Methyldopa treated patients were screened regularly for evidence of red cell autoantibody formation and samples were obtained from those with strongly positive IgG direct Coombs tests

Blood donors [3] These subjects were found to have positive IgG direct Coombs tests in the course of routine serological testing Each of them were healthy and haematologically normal

Red cell samples Blood samples were stored as clotted or EDTA or ACD anti coagulated specimens for up to 5 days at 4 °C before testing Preliminary studies showed that samples stored in this way did not alter significantly in reactivity in the phagocytosis system Immediately before assay red cells were washed 3 times in normal saline and suspended at a haematocrit of 5% in Hanks solution containing 10% fetal calf serum

IgG-sensitised red cell controls Aliquots of heparin anti coagulated red cells from the blood samples which provided normal monocytes for the same experiment

(see below) were washed 3 times in normal saline and incubated at 37 °C with equal volumes of dilutions of a standard Rhesus anti D antiserum This reagent comprised a pool of sera from four maternally iso-immunised ■ negative women After 30 min incubation the red cells were again washed 3 times in normal saline and suspended in Hanks' solution containing 10% fetal calf serum

Monocyte phagocytosis assay This technique is essentially identical to the method described by ABRAMSON *et al* [8] For each experiment venous blood was obtained from the same healthy normal subject and mixed with a half volume of 3% dextran in normal saline containing 10 units/ml of preservative free heparin After 1–1.5 h sedimentation at room temperature the white cells in the supernatant were washed 3 times in Hanks solution and suspended at a concentration of 6–8×10⁶/ml in Hanks solution containing 10% fetal calf serum Aliquots of the cell suspension (0.5 ml) were dispensed into 16 mm diameter flat bottomed plastic wells (Linbro Chemical Co., Code FB54) After 1 h at room temperature during which time cells sedimented to the floors of the wells, non adherent cells were removed by washing with two changes of Hanks solution Preliminary experiments established that the great majority of cells remaining after this step were monocytes and polymorphs The wells were then filled with 0.5 ml of red cell suspension each red cell sample being tested in duplicate The wells were sealed with parafilm and incubated at 37 °C for 2 75 h Non adherent red cells were removed by a single wash with Hanks solution followed by two washes in isotonic pH 7 4 buffered saline containing 2% bovine serum albumin The wells were air dried fixed for 5 min in methanol and Giemsa stained The number of red cells engulfed by 200 monocytes was counted for each well and the mean of each duplicate calculated

Calculation of strength of opsonisation Red cells sensitised with IgG were avidly phagocytosed by monocytes under the conditions of these experiments and the degree of phagocytosis was directly related to the concentration of sensitising antiserum This relationship is illustrated in figure 1

Red cell samples from DCT positive subjects were tested in batches of between one and four at a time in the phagocytosis assay It was necessary to compensate for possible between-experiment variations in the activity of monocyte monolayers prepared on different occasions This was achieved by including two IgG sensitised red cell controls in each experiment These controls consisted of red cells sensitised with two dilutions of the standard antiserum (0 125 and 0 03125) chosen because they had been shown to cause strong and weak phagocytosis, respectively (fig 1) The degree of phagocytosis of these two standards in any experiment was plotted on a log scale against the two serum dilutions, and a straight line drawn through these two points (giving a slope similar to that illustrated in figure 1) This graph was then used to convert phagocytosis figures for each DCT positive red cell sample tested in the same experiment into strength of opsonisation This value is the concentration of the standard antiserum which would have caused the same degree of phagocytosis if it were used to sensitise normal red cells for testing against the same monocyte monolayer

Elution of antibody from direct Coombs-positive red cells The acid elution technique described by KIDD [9] was used for this purpose The red cells had been stored in ACD at 4 °C before elution

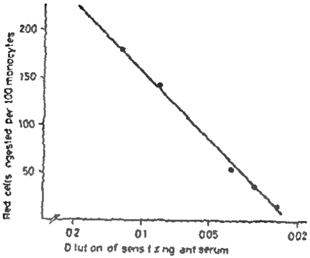


Fig 1 Relationship between the dilutions of anti D antiserum with which D positive red cells are coated and the strength of phagocytosis by normal monocytes

Autoanalyser testing of antibody potency The agglutination of red cells in an autoanalyser circuit, in the presence of antibody, polyvinyl pyrolidone and bromelin was used to assay relative antibody potency [10 11]

Results

Phagocytosis of direct Coombs-positive red cells The subjects who provided DCT-positive cell samples were divided into those in whom there was evidence of haemolysis (anaemia, reticulocytosis), and those who were haematologically normal The avidity with which red cells from the two groups were phagocytosed (strength of opsonisation) is shown in figure 2 It is apparent that red cells from non-haemolysing subjects were readily engulfed by monocytes (fig 3), and that the strength of phagocytosis in this group was no weaker than the reactions obtained with red cells from cases of immune haemolytic disease

Phagocytosis induced by elute from DCT-positive red cells Red cells were obtained from a blood donor who had been shown on regular testing over the course of more than 3 years to have a persistant strongly positive DCT Antibody was eluted from his red cells and shown to agglutinate normal red cells strongly by the indirect Coombs' test Aliquots of normal red cells were sensitised with four different dilutions of the eluate and

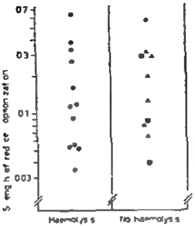


Fig 2 The readiness with which red cells from haemolysing and non haemolysing subjects were engulfed by normal monocytes (see text for method of calculating 'strength of red cell opsonisation') ● = Haemolytic anaemia (active), ♦ = haemolytic anaemia (quiescent) ▲ = methyldopa patients, ■ = normal blood donors

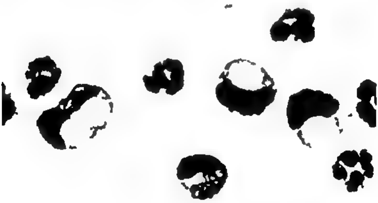


Fig 3 Red cells from a DCT positive healthy blood donor phagocytosed by normal monocytes Giemsa stain ×665

with four dilutions of the standard pooled anti D antiserum These sensitised cells were then tested in a single experiment with normal monocytes The eluate and standard antiserum were simultaneously tested against the same batch of red cells in the autoanalyser circuit

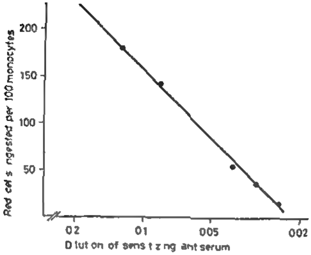


Fig 1 Relationship between the dilutions of anti D antiserum with which D positive red cells are coated and the strength of phagocytosis by normal monocytes

Autoanalyser testing of antibody potency The agglutination of red cells in an autoanalyser circuit in the presence of antibody, polyvinyl pyrolidone and bromelin was used to assay relative antibody potency [10, 11]

Results

Phagocytosis of direct Coombs-positive red cells The subjects who provided DCT-positive cell samples were divided into those in whom there was evidence of haemolysis (anaemia, reticulocytosis), and those who were haematologically normal The avidity with which red cells from the two groups were phagocytosed (strength of opsonisation) is shown in figure 2 It is apparent that red cells from non-haemolysing subjects were readily engulfed by monocytes (fig 3), and that the strength of phagocytosis in this group was no weaker than the reactions obtained with red cells from cases of immune haemolytic disease

Phagocytosis induced by elute from DCT-positive red cells Red cells were obtained from a blood donor who had been shown on regular testing over the course of more than 3 years to have a persistant strongly positive DCT Antibody was eluted from his red cells and shown to agglutinate normal red cells strongly by the indirect Coombs' test Aliquots of normal red cells were sensitised with four different dilutions of the eluate and

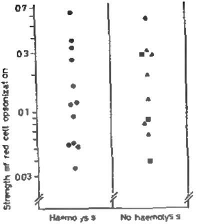


Fig 2 The readiness with which red cells from haemolysing and non haemolysing subjects were engulfed by normal monocytes (see text for method of calculating 'strength of red cell opsonisation') ● = Haemolytic anaemia (active), ♦ = haemolytic anaemia (quiescent), ▲ = methyldopa patients, ■ = normal blood donors

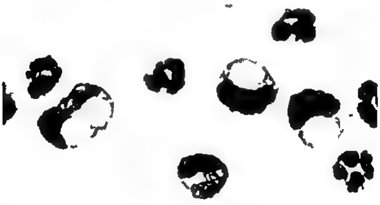


Fig 3 Red cells from a DCT positive healthy blood donor phagocytosed by normal monocytes Giemsa stain ×665

with four dilutions of the standard pooled anti-D antiserum These sensitised cells were then tested in a single experiment with normal monocytes The eluate and standard antiserum were simultaneously tested against the same batch of red cells in the autoanalyser circuit

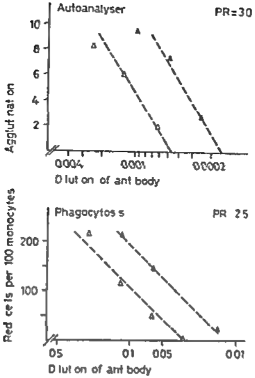


Fig 4 The relative agglutinating (as measured in the autoanalyser circuit) and opsonising (as measured by monocyte phagocytosis) potency of an anti D antiserum compared with autoantibody eluted from the red cells of a DCT-positive blood donor PR = Potency ratio Δ = Eluate, ▲ = anti-D

Figure 4 compares the relative potency of the two antibodies in the autoanalyser and phagocytosis system The eluate was approximately 2 5 times weaker than the anti-D antiserum in inducing phagocytosis and approximately 3 times weaker as an agglutinin

Discussion

The subclasses of IgG show considerable heterogeneity in their reactivity with the Fc receptors of mononuclear phagocytes Antibodies belonging to the classes IgG1 and IgG3 have been shown in a number of independent studies to bind considerably more strongly to Fc receptors than do IgG2 and IgG4 molecules [12–14] The fact that antibodies to the Rhesus-D antigen are predominantly in the IgG1 and IgG3 subclasses [15] appears to correlate well with the readiness with which these anti-

bodies cause accelerated red cell destruction *in vivo* Further support for this concept is found in the report by GELFAND *et al* [16] of a case of autoimmune haemolytic anaemia due to an IgG3 autoantibody in whom buffy coat erythro-phagocytosis could be demonstrated, and in the patient reported by GERGELY *et al* [17] with a positive direct Coombs' test due to a IgG4 antibody in whom there was no evidence of haemolysis ENGELFRIET [18] has studied two subjects whose red cells were coated with IgG2 autoantibody and a further individual with an IgG4 antibody In none of these cases was haemolysis demonstrable

Other findings, however, have raised the question of how closely the *in vitro* reactions of IgG antibodies reflect their haemolytic potential ENGELFRIET [18] reports that although IgG3 autoantibodies, in keeping with their strong binding to Fc receptors, almost always lead to accelerated red cell destruction, IgG1 red cell sensitisation on the other hand, which also produces striking phagocytosis *in vitro*, may be associated with normal red cell survival

The present results further support this concept that phagocytosis *in vitro* may not necessarily parallel the fate of red cells *in vivo*, in that erythrocytes from non-haemolysing subjects were readily ingested by normal monocytes In addition it was found that antibody eluted from the red cells of a DCT-positive healthy blood donor was as potent an opsonin *in vitro*, as it was an agglutinin If the strength of agglutination in the autoanalyser circuit is proportional to the number of IgG molecules carried per red cell, the latter experiment suggests that, molecule for molecule, 'harmless' auto-antibodies found in healthy subjects are as avidly bound by mononuclear cell Fc receptors as are haemolytic antibodies

The absence of haemolysis in DCT-positive subjects is explicable in several ways It may represent hitherto undiscovered characteristics of their IgG-coated red cells These features may be intrinsic to the antibodies themselves, or possibly involve accessory phenomena, such as the binding of additional factors to the red cell surface, or peculiarities of the erythrocyte membrane Whatever the characteristics of the antibody-coated cells which allow them to escape haemolysis, however, the monocyte phagocytosis assay appears not to recognise them

An alternative explanation is that these subjects fail to recognise and destroy IgG-sensitised red cells because of some abnormality in their phagocytosis mechanisms Survival studies of transfused IgG-sensitised red cells from these subjects in normal recipients could answer this question, although rapid elution of antibody when the cells enter the new envi-

ronment might possibly give spuriously prolonged survival Preliminary experiments using the monocyte assay have shown that monocytes from two of the blood donors in this series were fully capable of ingesting IgG-sensitised red cells, but this does not preclude abnormalities of recognition and phagocytosis in the spleen and liver in these individuals

Acknowledgements I am grateful to Mr P BOWELL, Dr M FISHER and Dr J GRANT for providing DCT-positive samples from normal blood donors and for the Rhesus antiserum, to Miss N JONES for samples from DCT positive patients, to Dr J MORTON who kindly assayed samples in the autoanalyser circuit, to Dr T PARRY who kindly photographed the figures, and to Dr M M PICKLES in whose laboratory this work was carried out

References

1 LOBUGLIO, A F, COTRAN, R S, and JANDL, J H Red cells coated with immunoglobulin G Binding and sphering by mononuclear cells in man Science *158* 1582–1585 (1967)

2 HUBER, H, DOUGLAS S D, and FUDENBERG, H H The IgG receptor an immunological marker for the characterisation of mononuclear cells Immunology *17* 7–21 (1969)

3 CLINE, M J and LEHRER, R I Phagocytosis by human monocytes Blood *32* 423–435 (1968)

4 ABRAMSON, N and SCHUR, P H The IgG subclasses of red cell antibodies and relationship to monocyte binding Blood *40* 500–508 (1972)

5 CARSTAIRS, K C, BRECKENRIDGE, A, DOLLERY, C T, and WORLLEDGE, S M Incidence of a positive direct Coombs test in patients on α methyldopa Lancet *ii* 133–135 (1966)

6 WEINER, W Coombs positive normal people Proc 10th Congr Int Soc Blood Transfus, Stockholm 1964, pp 35–39 (Karger, Basel 1965)

7 MOLLISON, P L Blood transfusion in clinical medicine, p 371 (Blackwell, Oxford 1972)

8 ABRAMSON, N, LOBUGLIO, A F, JANDL, J H, and COTRAN, R S The interaction between human monocytes and red cells binding characteristics J exp Med *132* 1191–1206 (1970)

9 KIDD, P Elution of an incomplete type of antibody from the erythrocytes in acquired haemolytic anaemia J clin Path *2* 103–108 (1949)

10 ROSENFIELD, K E and HABER, G V Detection and measurement of homologous human haemagglutinins Technicon Symposium on Automation in Analytical Chemistry, pp 503–506 (Mediad, New York 1965)

11 MORTON J A and PICKLES, M M Screening for blood group antibodies with the autoanalyser Technicon Symposium on Automation in Analytical Chemistry, pp 535–538 (Mediad, New York 1965)

12 ABRAMSON, N, GELFAND, E W JANDL, J H, and ROSEN F S The interaction

between human monocytes and red cells Specificity for IgG subclasses and IgG fragments J exp Med *132* 1207–1215 (1970)
13 HAY, F C, TORRIGIANI, G, and ROITT, I M The binding of human IgG sub classes to human monocytes Eur J Immunol 2 257–261 (1972)
14 HUBER, H, DOUGLAS, S D, NUSBACHER, J, KOCHWA, S, and ROSENFIELD, R E IgG subclass specificity of human monocyte receptor sites Nature, Lond *229* 419–420 (1971)
15 NATVIG J B, KUNKEL, H G, and LITWIN, S D Genetic markers of the heavy chain subgroups of human γG globulin Cold Spring Harb Symp quant Biol *32* 173–178 (1967)
16 GELFAND E W, ABRAMSON, N, SEGAL, G S, and NATHAN, D G Buffy coat observations and red cell antibodies in acquired haemolytic anaemia New Engl J Med *284* 1250–1252 (1971)
17 GERGELY, J, FUDENBERG, H H., and VAN LOGHEM E The papain susceptibility of IgG myeloma proteins of different heavy chain subclasses Immunochemistry *7* 1–6 (1970)
18 ENGELFRIET, C P Personal commun (1974)

Dr D Y MASON Department of Pathology, Gibson Laboratories Radcliffe Infirmary *Oxford OX2 6HE* (England)

Acta haemat. 55: 10–20 (1976)

Erythrocyte Enzymes in Neonatal Jaundice[1]

U. BIENZLE, C. E. EFFIONG, V. E. AIMAKU and L. LUZZATTO

Department of Haematology, Department of Paediatrics, and
Department of Obstetrics and Gynaecology, University College Hospital, Ibadan

Key Words. Erythrocyte enzymes · Glucose-6-phosphate dehydrogenase · Glutathione peroxidase · Glutathione reductase · Neonatal jaundice

Abstract. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a major cause of severe neonatal jaundice in Nigeria, but not all G6PD-deficient babies become jaundiced. Neonatal jaundice not attributable to G6PD deficiency nor to any other known aetiology is also common. In an effort to explain these two facts, we have measured the levels of the three enzymes G6PD, glutathione peroxidase (GSHPX) and glutathione reductase (GSSGR) in 38 jaundiced newborns, 26 control newborns and 44 normal adults, all of them males. We could not yet prove an additive effect of GSSGR or GSHPX deficiency with G6PD deficiency in causing jaundice. There was no evidence that low levels of GSHPX *per se* are associated with jaundice. However, jaundiced newborns with normal G6PD had significantly lower levels of GSSGR than control newborns with normal G6PD. These data suggest that a relatively low activity of GSSGR, a riboflavin-dependent enzyme, may predispose the red cells to accelerated destruction in the neonatal period.

Erythrocytes of newborns are generally more susceptible to haemolysis than those of adults, and it has been proposed that hydrogen peroxide may be an important cause of oxidative damage to these cells [2]. At least three enzymes are known to be involved in the detoxification of H_2O_2 in the erythrocyte: GSHPX[2], which directly destroys H_2O_2; GSSGR, which

[1] This work received financial support from USPHS grant GM 17261 and from the World ... am ... ENZLE was a Research Fellow sponsored by the Deutsc...

[2] The f ... used: ACD = a... dextrose; FAD = fla... reduced glutath... = oxidized glutath... GSSGR = ... reductase; G6P = ... phosph... nase; NADP = ni... N... uced NADP; 6PG =

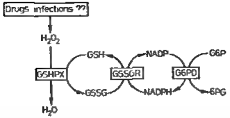


Fig 1 Main pathway of detoxification of hydrogen peroxide in human erythrocytes The disposal of H_2O_2 by the alternative catalase reaction is likely to be relatively ineffective at the expected intracellular concentration of H_2O_2 [5], especially in newborns who have in general catalase levels lower than adults [22]

regenerates the reduced glutathione consumed by GSHPX, and G6PD, which regenerates the NADPH consumed by GSSGR (fig 1) One could therefore expect that deficiency or decreased activity of one or more of those enzymes might lead to hyperbilirubinaemia in the newborn Indeed, genetically determined G6PD deficiency has been identified as an important cause of neonatal jaundice in Greece since 1961 [6] Genetically determined deficiency of GSHPX has also been associated with neonatal jaundice in some cases [20], and newborns with low levels of this enzyme may have an increased chance of developing severe degrees of hyperbilirubinaemia [30] Regarding GSSGR and its possible importance as an aetiological factor in the pathogenesis of neonatal jaundice, there is no information as yet in the literature to the best of our knowledge

In a preceding paper from this institution [8] the high prevalence of neonatal jaundice in Southern Nigeria has been documented, and G6PD deficiency has emerged as one of the major aetiological factors In this study we have specifically tested the possible role of the other two enzymes mentioned GSHPX and GSSGR, in the pathogenesis of hyperbilirubinaemia in the newborn, and their possible interactions with G6PD itself It will be shown that low levels of GSSGR correlate significantly with the development of jaundice

Subjects and Methods

G6PD GSHPX and GSSGR were tested in three groups of subjects (1) 38 male newborns 3 of them prematures with birth weights below 2 500 g, admitted to the

[illegible] Hospital Ibadan because of jaundice [illegible], (2) 26 normal male newborns de[illegible] group of 44 adult blood donors

[illegible] a combination of the following tech[illegible] according to Porter *et al* [24] with slight modifi[illegible] according to the recommendations of WHO [illegible] [11]

[illegible] according to Paglia and Valentine [23] *Blood* [illegible] with cold isotonic NaCl solution the [illegible] deionised H_2O 0 005 *ml* of haemolysate [illegible] strength Drabkin solution was added to [illegible] of 2 5 ml of 0 05 M phosphate buffer pH 7 1 [illegible] NADPH 0 1 ml of 0 15 M GSH 0 01 ml [illegible] purified GSSGR (Boehringer) After 5 min [illegible] *the reaction was started by adding 0 02 ml of* [illegible] of optical density at 340 nm was fol[illegible] absorbance recorder At the same time [illegible] of GSH was measured by adding the same amount of H_2O_2 [illegible] of haemolysate and the resulting slope was [illegible] reaction

GSSGR [illegible] was measured according to Horn [15] The components of the [illegible] *were always added in the following sequence* 0 7 ml of 0 067 M *phos*phate buffer pH 6 6 0 2 ml of 7 5 mM *GSSG* 0 05 [illegible] 6 mM NADPH and the reaction was started by addition of 0 05 ml of hae[illegible] In a number of cases haemolysates of newborns were incubated for 1 [illegible] emperature with FAD to give a f[illegible] oncentration of 7 µM befo[illegible] GSGR was per formed

All enzyme acti[illegible] expre[illegible] interna[illegible] haemoglo*bin*

Statistical analy[illegible] data [illegible] ilcoxon rank test

Glucose 6-ph[illegible] *hydrog*[illegible]
diced babies) 17 [illegible] re fou[illegible]
(55%) had norm[illegible] vity ([illegible]

[a] It was not po[illegible] series to
jaundice were of h[illegible] in Th[illegible]
the basis of the ha[illegible] e reticu[illegible]
haemolysis from tl[illegible] ork in
criteria for this pu[illegible] the
babies with non h[illegible]
possible association[illegible]

Table I High prevalence of G6PD deficiency in babies with neonatal jaundice in Nigeria

	G6PD deficiency, %
Normal adults [17]	21.6
Normal newborns [NYLANDER and LUZZATTO, unpublished results]	22.5
Newborns with neonatal jaundice [8]	51.0
Newborns with neonatal jaundice [this study]	45.0
Newborns with severe neonatal jaundice (serum bilirubin > 18 mg/100 ml) [this study]	78.5

Table II Statistical analysis of differences in levels of GSSGR

G6PD status	Comparison	p level
Normal	normal newborns *vs* adults	<0.01
Deficient	jaundiced newborns *vs* normal newborns	>0.10
Normal	jaundiced newborns *vs* normal newborns	<0.02

Table III Effect of FAD on GSSGR activity in haemolysates from newborns

G6PD status	GSSGR activity, IU/g Hb (mean ± SD)		Stimulation, %
	no addition	+7 μM FAD	
Normal (n = 7)	12.0 ± 2.3	23.0 ± 4.5	[illegible]
Deficient (n = 5)	19.8 ± 2.9	26.5 ± 3.7	35

and 2 were G6PD normal) (table I). The proportions are in good agreement with the data reported by EFFIONG *et al.* [8] on a larger series by a screening method alone. Electrophoretic characterization of the enzyme from G6PD normal babies showed that 15 had type B and 6 had type A, in agreement with the distribution of G6PD variants observed by LUZZATTO and ALLAN [17] in adults, and by NYLANDER and LUZZATTO [unpublished results] in newborns (G6PD type B 55.5%, type A 22.9%, type A⁻ 21.6%).

Glutathione peroxidase. A wide scatter in the values of activity was observed in all three groups studied (fig. 2). GSHPX levels are signifi-

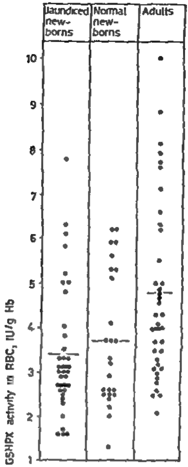


Fig 2 GSHPX levels in the three groups of subjects studied Each dot represents one subject Horizontal bars represent arithmetical means

cantly higher in adults than in newborns with or without jaundice, but there is extensive overlap in the distribution The average activity of GSHPX is lower in babies with neonatal jaundice than in normal newborns, but the difference between these two groups does not reach significance *The distribution of enzyme activities of G6PD-normal and G6PD-deficient subjects does not differ significantly if each group is considered separately*

Glutathione reductase Since GSSGR is increased in G6PD deficiency [27], the data on this enzyme are presented separately for G6PD-normal and G6PD-deficient subjects (fig 3) As expected, in all three groups G6PD-deficient subjects have significantly higher GSSGR levels than

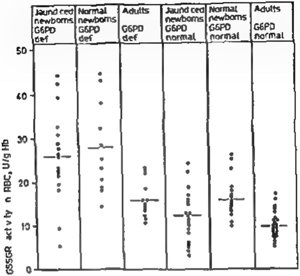


Fig 3 GSSGR levels in the three groups of subjects studied subdivided according to G6PD status Each dot represents one subject Horizontal bars represent arithmetical means

G6PD normal individuals The average GSSGR values were significantly higher in both groups of newborns than in adults, as has been previously demonstrated for premature and normal babies by GROSS *et al* [13] When jaundiced and normal newborns are compared, the former are found to have lower GSSGR activities The difference does not reach significance in G6PD deficient newborns, but it is significant in G6PD normal babies (table II) No significant correlation was found between gestational age, birth weight and GSSGR and GSHPX activity in G6PD-normal and G6PD-deficient babies with and without jaundice We also could not detect any relationship between the clinical picture, serum bilirubin levels and enzyme activities in the patients with neonatal jaundice

Stimulation of GSSGR by FAD The coenzyme of GSSGR is FAD, and dietary intake of riboflavin or addition of FAD in minute amount to haemolysates increases the activity of GSSGR [1] In 13 newborns (with or without jaundice) haemolysates were assayed for GSSGR before and after preincubation with FAD The percent increase in GSSGR activity is much higher in haemolysates with low initial GSSGR activity (table III) Thus, after addition of FAD, the absolute GSSGR activity is very similar

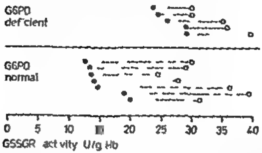


Fig 4 Effect of 7 μM FAD on GSSGR activity of crude haemolysates See data of table III For each subject the full circle represents the activity without added FAD and the open circle connected to the full circle by a broken line represents the activity for the same subject with added FAD

in the two groups of haemolysates It appears that G6PD deficient red cells, rather than having more GSSGR, have GSSGR which is already nearly saturated with FAD (fig 4) [19]

Discussion

EFFIONG *et al* [8] have demonstrated the major role of G6PD deficiency in the pathogenesis of neonatal jaundice in Nigeria, in agreement with previous reports on smaller series [12, 14, 25] Since we found that all of the G6PD deficient jaundiced newborns had the A⁻ type of this enzyme, this variant must be included, together with the Mediterranean variant [6] and with those encountered in the Far East [19] amongst those which increase the risk of clinically significant haemolysis in the perinatal period EFFIONG *et al* [8] also pointed out that, apart from ABO incompatibility (less than 20% of our patients), the pathogenesis of neonatal jaundice remains obscure in a substantial proportion of cases In the present study we were therefore concerned mainly with two specific questions

(1) Since not all G6PD deficient babies develop jaundice, what is the additional factor, or factors responsible for this complication? It appeared possible that the jaundiced babies might also have, in addition to genetically determined G6PD deficiency, perhaps transiently and on a non genetic basis a low level of either GSSGR or GSHPX Indeed this was found to be true for both enzymes, but with the number of cases presently available, the differences observed are not statistically significant

Thus, the notion that the additive effect of two enzyme abnormalities might be a cause of neonatal jaundice, remains a hypothesis which we are continuing to test

(2) Since GSSGR and GSHPX, in addition to G6PD, are both involved in detoxification of hydrogen peroxide, it is possible that low levels of either or both of these enzymes are associated with the development of otherwise unexplained neonatal jaundice As far as GSHPX is concerned, although NECHELES *et al* [20] have reported association of partial deficiency of this enzyme with haemolytic disease in neonates, we could not find evidence so far that this plays a role in the pathogenesis of neonatal jaundice in Nigeria We also could not demonstrate any correlation between GSHPX and GSSGR activity in individuals of any of the three groups studied On the other hand, while there does not seem to be any reference to the possible role of GSSGR in the development of neonatal haemolysis, our data show clearly that the levels of this enzyme in jaundiced babies are lower than in normal babies, once both groups are matched with respect to their G6PD status On theoretical grounds, this finding is not surprising, since a number of workers [3, 4, 10, 16] have already suggested that low levels of GSSGR can be associated with clinically important haemolysis

Based on the number of cases presently available, the difference in GSSGR levels is not statistically significant amongst G6PD deficient babies, but it is significant at the 2% level in the G6PD normal babies Since G6PD deficient subjects have, as a rule, higher GSSGR, it is conceivable that these two enzymes interact in a peculiar way with respect to causing neonatal jaundice paradoxically, G6PD deficiency might protect a baby against developing a dangerously low level of GSSGR It is not likely that low GSSGR is an effect, rather than a cause, of neonatal jaundice, since the activity of this enzyme is higher in young that in old fetal erythrocytes [29], and haemolysis would therefore, if anything, raise its level in whole blood

These results suggest that GSSGR may be important in the pathogenesis of severe neonatal jaundice in Nigeria The incidence of 'deficiency' of this enzyme will have to be established by testing an unselected group of newborns However, it would not be surprising if it was relatively common, since it is now known to occur rarely, if ever, on a genetic basis, rather, it usually results from a low dietary intake of riboflavin [7], and suboptimal riboflavin levels are prevalent in Southern Nigeria For instance, 37% of a group of pregnant women exhibited very low urinary ri-

boflavin excretion values consistent with vitamin deficiency [21] The ratio of activity of GSSGR with and without added FAD is itself a sensitive index of the riboflavin status in man [26 28] and the fact that the FAD stimulation observed by us (fig 4) is much more marked than reported on cord bloods in USA [32] is consistent with the possibility that many of our newborns have suboptimal levels of riboflavin Treatment with riboflavin of GSSGR-'deficient subjects increases the enzyme activity to normal or above normal levels [1 28] We can thus entertain the contention that administration of riboflavin to pregnant women near term may help to prevent neonatal jaundice in at least some cases in this community

Acknowledgements We are grateful to all our colleagues in the Departments of Paediatrics and Obstetrics and Gynaecology who have cooperated with this study We also thank Mr F A Fasuan and Mr V C N Okoye for technical assistance and the Medical Illustration Unit for preparing the figures

References

1 Beutler E Effect of flavin compounds on glutathione reductase activity *In vivo* and *in vitro* studies J clin Invest *48* 1957 (1969)

2 Bracci R Seeler R Rudolph N Kochen J, and Gross R T Erythrocyte glutathione peroxidase activity and hydrogen peroxide sensitivity a mechanism for drug induced hemolysis in the newborn J Pediat *67* 938 (1965)

3 Brittinger G Löhr G and König E Angeborene nichtsphärozytäre hämolytische Anämie bei familiärem Mangel an DPNH und TPNH abhängiger Glutathionreduktase der Erythrocyten Klin Wschr *43* 427 (1965)

4 Carson P E Brewer G J, and Ickes C E Decreased glutathione reductase with susceptibility to hemolysis J Lab clin Med *58* 804 (1961)

5 Cohen G and Hochstein P Glutathione peroxidase the primary agent for the elimination of hydrogen peroxide in erythrocytes Biochemistry N Y 2 1420 (1963)

6 Doxiadis S A Fessas P Valaes T and Mastrokalos N Glucose 6 phosphate dehydrogenase deficiency a new aetiological factor of severe neonatal jaundice Lancet *i* 297 (1961)

7 Editorial Erythrocyte glutathione reductase a measure of riboflavin nutritional status Nutr Rev *30* 162 (1972)

8 Effiong C E Aimaku V E Bienzle U Oyedeji G A Ikezi G and Ikpe D E Neonatal jaundice in Ibadan incidence and aetiological factors in babies born in hospital (submitted for publication)

9 Flatz G Enhanced binding of FAD to glutathione reductase in G6PD deficiency Nature Lond *226* 755 (1970)

10 FORNAINI, G, BIANCHINI, E, LEONCINI, G, and FANTONI, A Metabolic aspects of red cells in congenital nonspherocytic haemolytic anaemia Br J Haemat *10* 23 (1964)

11 GALL, J C, BREWER, G J, and DERN, R J Studies of glucose 6 phosphate dehydrogenase activity of individual erythrocytes the methemoglobin elution test for identification of females heterozygous for G6PD deficiency Am J hum Genet *17* 359 (1965)

12 GILLES, H M and ARTHUR, C J [redacted] Erythrocyte enzyme deficiency in unexplained neonatal jaundice W Afr med J *9* 266 (1960)

13 GROSS, [redacted] T, BRACCI, R, RUDOLPH, N, SCHROEDER, E, and KOCHEN, J A Hydrogen peroxide toxicity and detoxification in the erythrocytes of newborn infants Blood *29* 481 (1967)

14 HENDRICKSE, R G Problems and future of paediatrics in preindustrial countries Africa Helv paediat Acta, suppl 28, p 23 (1972)

15 HORN, M D in BERGMEYER Methoden der enzymatischen Analyse, p 875 (Chemie Verlag, Weinheim 1962)

16 LOHR G W und WALLER, H D Eine neue enzymopenische, hämolytische Anämie mit Glutathionreduktase Mangel Med sche Klin *57* 1521 (1962)

17 LUZZATTO, L and ALLAN, N C Relationship between the genes for glucose 6 phosphate dehydrogenase and for haemoglobin in a Nigerian population Nature, Lond *219* 1041 (1968)

18 LUZZATTO, L and AFOLAYAN, A Enzymic properties of different types of human erythrocyte glucose 6 phosphate dehydrogenase, with characterization of two new genetic variants J clin Invest *47* 1833 (1968)

19 LUZZATTO, L New developments in glucose 6 phosphate dehydrogenase deficiency Israel J Med Scis *9* 1484 (1973)

20 NECHELES, T F, BOLES, T A, and ALLEN, D M Erythrocyte glutathione peroxidase deficiency and hemolytic disease of the newborn infant J Pediat *72* 319 (1968)

21 Nutritional Survey, Republic of Nigeria, p 98 (1965)

22 OSKI, F A and NAIMAN, J L Hematologic problems in the newborn, p 75 (Saunders, Philadelphia 1966)

23 PAGLIA, D E and VALENTINE, W N Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase J Lab clin Med *70* 158 (1967)

24 PORTER I H, BOYER, S H, WATSON WILLIAMS, E J, ADAM, A, SZEINBERG, A, and SINISCALCO, M Variation of glucose 6-phosphate dehydrogenase in different populations Lancet *i* 895 (1964)

25 RANSOME KUTI, O The problem of paediatric emergencies in Nigeria Niger med J *2* 62 (1972)

26 SAUBERLICH, H E, JUDD, J H, NICHOLAS, [redacted] E, BROQUIST, P H, and DARBY, W J Application of the erythrocyte glutathione reductase assay in evaluating riboflavin nutritional status in a high school student population Am J clin Nutr *25* 756 (1972)

27 SCHRIER, S L, KELLERMAYER, R W, CARSON, P E, ICKES, C E, and ALVING

A S The hemolytic effect of primaquine IX Enzymatic abnormalities in primaquine sensitive erythrocytes J Lab clin Med 52 109 (1958)

28 TILLOTSON J A and BAKER E M An enzymatic measurement of riboflavin status in man Am J clin Nutr 25 425 (1972)

29 VETRELLA M BARTHELMAI W und RIETKOTTER J Aktivitat der Glutathion Peroxidase in Erythrozyten vom Fetal bis zum Erwachsenenalter Klin Wschr *48* 85 (1970)

30 WHAUN J M and OSKI F A Relation of red blood cell glutathione peroxidase to neonatal jaundice J Pediat *76* 555 (1970)

31 WHO Standardization of procedures for the study of glucose 6 phosphate dehydrogenase Tech Rep Ser Wld Hlth Org No 366 (1967)

32 YAWATA Y and TANAKA K E Activation of glutathione reductase by flavine adenine dinucleotide in human adult and cord red cells Experientia *27* 781 (1971)

Dr U BIENZLE Department fur Kinderheilkunde Universitat Ulm Prittwitzstr 43 *D-7900 Ulm/Donau* (BRD)

Acta haemat 55 21-27 (1976)

Blood Coagulation in Patients with Acute Infectious Hepatitis in India

H Dube, J P Gupta, D S Singh, V N Sinha, Sulekha Bhattacharya and Ramakanta Dube

Institute of Medical Sciences, Banaras Hindu University, Varanasi

Key Words Bleeding disorders Blood coagulation Hepatitis Liver diseases

Abstract Coagulation studies were performed in 61 patients of acute infective hepatitis 18 with clinical signs of liver failure had bleeding and all succumbed The 47 patients without liver failure showed no haemorrhagic diathesis and all of them had uneventful recovery Though coagulopathy was present in most of the patients the severity and frequency of coagulation defects were more in those with signs of hepatic failure Hypofibrinogenemia, elevated serum fibrinogen degradation products and accelerated euglobulin lysis were conspicuous in patients with hepatic failure It appears that while diminished synthesis of coagulation factors is the main basis for coagulopathy in patients without hepatic failure, additional factors like local or disseminated intravascular coagulation and increased fibrinolysis also contribute significantly to the coagulopathy in cases of liver failure

Blood coagulation abnormalities in liver disorders are frequent since liver plays important roles in synthesis of coagulation factors [11] and in clearance of activated clotting factors [5] and plasminogen activator [7] from the circulation However, though many studies on chronic liver disease particularly hepatic cirrhosis are available [6, 17] observations on acute hepatocellular disease are conspicuously few Only recently an exhaustive study has appeared from Australia [8] The present paper describes our observations on the coagulation profile of 61 patients of infective hepatitis in India

Material and Methods

The 61 patients included in this series were either in patients of Medical, Obstetrics and Gynaecology and Student Wards, or out patients from Students' Clinic of

SSL Hospital Banaras Hindu University They were investigated during an epidemic outbreak of infective hepatitis during the winters of 1972–73 The diagnosis was based on clinical history, physical examination and corroborated by liver function tests The patients were divided into two groups (1) 43 patients (34 males and 9 females) without signs of liver failure and (2) 18 patients (14 females and 4 males) with signs of liver failure, as determined by mental changes, flapping tremor and fetor hepaticus [8] 12 of them were in coma grade IV and the remaining 6 in coma grade III [18] The patients were investigated within 1–2 weeks of clinical appearance of jaundice, the serum bilirubin ranging between 2 and 15 mg% at the time of investigation

Routine haematological tests were done as described by DACIE and LEWIS [2] Coagulation tests were mostly done as described by DENSON [3] The other techniques used were plasma fibrinogen [13] euglobulin lysis time [1] Astrup fibrin plate lysis as described by DACIE and LEWIS [2] using bovine fibrinogen (Sigma) thrombin time [12] serum FDP [10] and PF 3 availability [9]

Results

Clinical haemorrhagic diathesis in these patients was conspicuously related with signs of hepatic failure All the patients with liver failure had clinical bleeding (table I) and all ultimately succumbed to the disease, while none of the patients without signs of liver failure showed clinical bleeding diathesis The latter group of patients uniformly had uneventful recovery Blood coagulation disturbances, however, occurred irrespective of the signs of hepatic failure (table II) Statistical analysis indicated significant prolongation of kaolin cephalin clotting time (KCCT), prothrombin time (PT) and thrombin time (TT) in both groups of patients

Table I Bleeding symptoms in infective hepatitis patients with hepatic failure

Bleeding symptoms	Females (n = 14)	Males (n = 4)
Petechiae	1	0
Ecchymoses	2	1
Bleeding from gums	2	0
Haematuria	4	0
Haematemesis	9	4
Post partum haemorrhage	6	–
Ante-partum haemorrhage	3	–

Factor II, V and VII+X were assayed in 18 patients without hepatic failure These factors were reduced in 40, 57 and 61% of patients respectively The lowest value of factor II was 25% in one patient Factors V and VII+X were less than 10% in 4 patients (22%)

The platelet changes were of a mild nature only, the lowest platelet count recorded was 120,000/μl in one case Platelet factor 3 (PF 3) availability index of less than 25% was noted in 4 patients, all of whom had hepatic failure Platelet aggregation was grossly abnormal in one patient with liver failure where the platelets completely failed to aggregate with adenosine diphosphate and epinephrine and where no PF 3 activation occurred with kaoline Statistically, the reduction of platelet count was significant in only those with hepatic failure but PF 3 availability index was significantly low in both groups of patients Plasma fibrinogen was not significantly altered in patients without liver failure In contrast, half of the patients with liver failure had plasma fibrinogen levels less than 150 mg% Statistically also there was significant hypofibrinogenaemia in this group Significant increase of fibrinolytic activity was detected in these patients The patients with and without liver failure had mean euglobulin lysis time (ELT) of 155 and 200 min, respectively (control 220 min), and the respective mean figures for the lysis zone on the Astrup fibrin plate caused by euglobulin fraction were 40 and 33 mm^2 (control 18 mm^2) Serum fibrinogen degradation products (FDP) were statistically elevated to a significant degree in both groups of patients, however, levels more than 20 μg/ml were only noted in 9 patients with hepatic failure Statistical analysis showed that all the tests, except Astrup plate lysis, were significantly more deranged in patients with hepatic failure as compared to patients without hepatic failure

Discussion

The high proportion of female patients with hepatic failure in this series is partly due to the treatment of uncomplicated female patients as out patients who were not investigated Secondly, there appears to be a true increase of susceptibility of pregnant patients to develop hepatic failure

The majority of the patients with acute infective hepatitis had blood coagulation abnormalities of varying degree The defects were more pronounced and more frequent in cases with liver failure as compared to those

Table II Coagulation parameters in infective hepatitis

	Kaolin cephalin clotting time, sec	Prothrombin time, ratio	Thrombin time, ratio	Platelets $\times 10^3/\mu l$
Group I patients without liver failure				
Range	35–177	1.0–2.3	1.0–2.0	120–400
Mean±SD	76±27**	1.3±0.34**	1.3±0.26**	260±50
	(n=43)	(n=43)	(n=24)	(n=43)
Group II patients with liver failure				
Range	67–α	1.1–α	1.1–α	160–350
Mean±SD	145±44**++	2.4±2.05**++	2.3±0.83**+++	220±40*++
	(n=18)	(n=18)	(n=18)	(n=18)
Control				
Range	40–65	–	–	220–500
Mean±SD	56±10			264±75
	(n=75)			(n=19)

* Mark indicates the statistical comparison of values with control
\+ Mark indicates the statistical comparison of values with group I

without liver failure. Clinically too the bleeding was manifest in patients with liver failure only. These observations are in complete agreement with the experience of GALLUS *et al.* [8].

Thrombocytopenia was not an important feature in patients with infective hepatitis. Only in 3 out of 61 cases, platelet counts were 150,000/μl or less. Platelet aggregation with ADP and PF 3 activity too did not exhibit any remarkable abnormality in patients without liver failure. PF 3 availability was significantly poor (less than 25% availability index) in 4 patients, all with liver failure.

The patients without liver failure showed lesser degree and frequency of prolongation of prothrombin time, KCCT and thrombin time. Factors II, V and VII+X estimated in 18 patients showed corresponding decreases in 40, 57 and 61% of cases respectively. Previous studies in infective

Table II (continued)

PF 3, %	Factor I mg%	Serum FDP µg/ml	Euglobulin lysis time min	Fibrin plate lysis, mm²
25 100 72±25 5** (n=23)	168–600 302±103 (n=43)	2 5–20 7±4 8** (n=23)	60–360 200±72 4* (n=43)	16–64 33±11 5** (n=23)
0–100 40±22 8**++ (n=14)	90–375 199±103**++ (n=18)	5–80 38±33 7**++ (n–18)	76–240 155±45 6**++ (n=18)	16–56 40±11 2** (n=14)
–	270–510 304±84 7 (n=19)	0–5 1 6±2 25 (n=10)	180–375 220±48 (n=20)	0–42 18±10 8 (n=15)

* and + p=0 05, ** and ++ p–0 01, +++ p=0 001

hepatitis [4, 8, 16] showed a similar reduction of these coagulation factors Plasma fibrinogen was mildly lower (150–200 mg%) in only 5 (11 5%) cases *Conspicuous increase of fibrinolytic activity was notable* only in a small percentage of patients, i e, euglobulin lysis time less than 2 h was seen in 5 cases and Astrup fibrin plate lysis zone more than 50 mm² occurred in only 2 patients FDP were not beyond 10 µg/ml in any patient The prolongation of prothrombin time and reduction of coagulation factors II, V and VII+X in patients without liver failure would appear, therefore, to be resulting mostly from the diminished synthesis of these factors in the damaged liver

The blood coagulation disturbances were uniformly more severe in patients with liver failure All of them had prolonged *prothrombin time and* KCCT and about two thirds of them showed hypofibrinogenaemia 28%

of them showed definite acceleration of plasma euglobulin lysis and 50% showed elevated levels of serum FDP. Gallus *et al.* [8] observed greatly reduced plasminogen levels in most patients and lowered plasma fibrinogen in over half of their patients. Rake *et al.* [14] reported reduced fibrinogen survival in patients with viral hepatitis and liver failure and attributed it to disseminated intravascular coagulation. In another paper Rake *et al.* [15] reported highly encouraging results with early administration of heparin in patients with liver failure. It appears that the pathogenesis of blood coagulation defects in these cases (with liver failure) is multifactorial, diminished hepatic synthesis of coagulation factors, elevated fibrinolytic activity and local/disseminated intravascular coagulation playing the contributory role to varying extent.

Acknowledgements. The authors are grateful to the Indian Council of Medical Research for the financial assistance for part of this work. We are thankful to Shri R. A. Tripathi and Shri R. M. Mishra, Laboratory Technicians, for their technical assistance.

References

1 Buckell, M.: The effect of citrate on euglobulin methods of estimating fibrinolytic activity. J. clin. Path. *11*: 403 (1958).

2 Dacie, J. V. and Lewis, S. M.: Practical haematology (Churchill, London 1968).

3 Denson, K. W. E.: Reagents and techniques; in Biggs and Macfarlane Treatment of haemophilia and other coagulation disorders (Blackwell, Oxford 1966).

4 Deutsch, E.: Blood coagulation changes in liver diseases; in Popper and Schaffner Progress in liver diseases. Grune and Stratton, New York, London *2*: 69–83 (1965).

5 Deykin, D.; Cochios, F.; Decamp, G., and Lopez, A.: Hepatic removal of activated factor X by the perfused rat liver. Am. J. Physiol. *214*: 414–419 (1968).

6 Donaldson, G. W. K.; Davies, S. H.; Darg, A., and Richmond, J.: Coagulation factors in chronic liver disease. J. clin. Path. *22*: 199–204 (1969).

7 Fletcher, A. P.; Biederman, O.; Moore, D.; Alkjaersig, N., and Sherry, S.: Abnormal plasminogen plasmin system activity (fibrinolysis) in patients with hepatic cirrhosis: its cause and consequences. J. clin. Invest. *43*: 681–695 (1964).

8 Gallus, A. S.; Lucas, C. R., and Hirsh, J.: Coagulation studies in patients with acute infectious hepatitis. Br. J. Haemat. *22*: 761–771 (1972).

9 Hardisty, R. M. and Hutton, R. A.: The kaolin clotting time of platelet rich plasma. A test of platelet factor 3 availability. Br. J. Haemat. *11*: 258–268 (1965).

10 MERSKEY, C, LALEZARI, P., and JOHNSON, A J A rapid, simple, sensitive method for measuring fibrinolytic split products in human serum Proc Soc exp Biol Med *131* 871–875 (1969)
11 OLSON, J P, MILLER, L L, and TROUP, S B Synthesis of clotting factors by the isolated perfused rat liver J clin Invest *45* 690–701 (1966)
12 OWEN, C A, jr, BOWIE, E J W, DIDISHEIM, P, and THOMPSON, J H The diagnosis of bleeding disorders (Churchill, London 1969)
13 QUICK, A J Hemorrhagic diseases and thrombosis (Lea & Febiger, Philadelphia 1966)
14 RAKE, M O, FLUTE, P T, PANNELL, G, and WILLIAMS, R Intravascular coagulation in acute hepatic necrosis Lancet *i* 533–536 (1970)
15 RAKE, M O, SHILKIN, K B, WINCH, J, FLUTE, P T; LEWIS, M L, and WILLIAMS, R Early and intensive therapy of intravascular coagulation in acute liver failure Lancet *ii* 1215–1218 (1971)
16 RATNOFF, O D Hemostatic mechanism in liver disease Med Clins N Am *47* 721–736 (1963)
17 SPECTOR, I and CORN, M Laboratory tests of haemostasis The relation to haemorrhage in liver disease Archs intern Med *119* 577–582 (1967)
18 TREY, C, LIPWORTH L, and DAVIDSON, C S Worldwide fulminant hepatic failure surveillance study Gastroenterology *58* 307 (1970)

Prof B DUBE, 1, Medical Enclave, Banaras Hindu University, *Varanasi* (India)

of them showed definite acceleration of plasma euglobulin lysis and 50% showed elevated levels of serum FDP. GALLUS *et al.* [8] observed greatly reduced plasminogen levels in most patients and lowered plasma fibrinogen in over half of their patients. RAKE *et al.* [14] reported reduced fibrinogen survival in patients with viral hepatitis and liver failure and attributed it to disseminated intravascular coagulation. In another paper RAKE *et al.* [15] reported highly encouraging results with early administration of heparin in patients with liver failure. It appears that the pathogenesis of blood coagulation defects in these cases (with liver failure) is multifactorial, diminished hepatic synthesis of coagulation factors, elevated fibrinolytic activity and local/disseminated intravascular coagulation playing the contributory role to varying extent.

Acknowledgements. The authors are grateful to the Indian Council of Medical Research for the financial assistance for part of this work. We are thankful to SHRI R. A. TRIPATHI and SHRI R. M. MISHRA, Laboratory Technicians, for their technical assistance.

References

1 BUCKELL, M.: The effect of citrate on euglobulin methods of estimating fibrinolytic activity. J. clin. Path. *11*: 403 (1958).
2 DACIE, J. V. and LEWIS, S. M.: Practical haematology (Churchill, London 1968).
3 DENSON, K. W. E.: Reagents and techniques, in BIGGS and MACFARLANE Treatment of haemophilia and other coagulation disorders (Blackwell, Oxford 1966).
4 DEUTSCH, E.: Blood coagulation changes in liver diseases, in POPPER and SCHAFFNER Progress in liver diseases. Grune and Stratton, New York, London *2*: 69–83 (1965).
5 DESKIN, D., COCHIOS, F., DECAMP, G., and LOPEZ, A.: Hepatic removal of activated factor X by the perfused rat liver. Am. J. Physiol. *214*: 414–419 (1968).
6 DONALDSON, G. W. K., DAVIES, S. H., DARG, A., and RICHMOND, J.: Coagulation factors in chronic liver disease. J. clin. Path. *22*: 199–204 (1969).
7 FLETCHER, A. P., BIEDERMAN, O., MOORE, D., AKJAERSIG, N., and SHERRY, S.: Abnormal plasminogen-plasmin system activity (fibrinolysis) in patients with hepatic cirrhosis: its cause and consequences. J. clin. Invest. *43*: 681–695 (1964).
8 GALLUS, A. S., LUCAS, C. R., and HIRSH, J.: Coagulation studies in patients with acute infectious hepatitis. Br. J. Haemat. *22*: 761–771 (1972).
9 HARDISTY, R. M. and HUTTON, R. A.: The kaolin clotting time of platelet rich plasma: A test of platelet factor-3 availability. Br. J. Haemat. *11*: 258–268 (1965).

10 Merskey, C., Lalezari, P., and Johnson, A J: A rapid, simple, sensitive method for measuring fibrinolytic split products in human serum Proc Soc exp Biol Med 131 871–875 (1969)
11 Olson, J P, Miller, L. L., and Troup, S B Synthesis of clotting factors by the isolated perfused rat liver J clin Invest 45 690–701 (1966)
12 Owen, C. A, jr, Bowie, E J W, Didisheim, P., and Thompson, J H The diagnosis of bleeding disorders (Churchill, London 1969)
13 Quick, A J Hemorrhagic diseases and thrombosis (Lea & Febiger, Philadelphia 1966)
14 Rake, M O, Flute, P T, Pannell, G, and Williams, R Intravascular coagulation in acute hepatic necrosis Lancet i 533–536 (1970)
15 Rake, M O, Shilkin, K B, Winch, J, Flute, P. T, Lewis, M L., and Williams R Early and intensive therapy of intravascular coagulation in acute liver failure Lancet ii 1215–1218 (1971)
16 Ratnoff, O D Hemostatic mechanism in liver disease Med Clins N Am 47 721–736 (1963)
17 Spector, I and Corn, M Laboratory tests of haemostasis The relation to haemorrhage in liver disease Archs intern Med 119 577–582 (1967)
18 Trey, C, Lipworth, L., and Davidson, C. S Worldwide fulminant hepatic failure surveillance study Gastroenterology 58 307 (1970)

Prof B Dube, 1 Medical Enclave Banaras Hindu University, Varanasi (India)

Acta haemat 55 28–35 (1976)

Involvement of the Central Nervous System in Rats with Acute Leukemia L 5222[1]

W Calvo and D Hoelzer

Department of Clinical Physiology Clinical Research Center University of Ulm
Ulm

Key Words Central nervous system hemorrhages · Leukemia · Meningeal leukemic infiltration · Rat leukemia L 5222

Abstract Young adult BD IX rats made leukemic by intravenous infusion of 2×10^7 nucleated blood cells from leukemic donors (L 5222) showed lesions in the central nervous system 6 days after transfer when their leukocyte count was between 250 000 and 350 000 cells/µl The brain was affected in only half of the animals whereas all showed an infiltration of the meninges Furthermore all animals had extensive lesions in the spinal cord The lesions consisted of nodules of leukemic cells and hemorrhages which were predominant in the lower half of the spinal cord and more abundant in the white than in the gray matter Paraplegia appeared in some animals shortly before death Since there is a consistent involvement of the meninges and spinal cord the L 5222 leukemia seems to be a useful model for the study of leukemic infiltration of the central nervous system and the reaction to chemotherapy

Leukemic involvement of the central nervous system particularly of the meninges, constitutes a neurological complication in human acute leukemia During a study of the growth characteristics of a transplantable acute leukemia in rats (L 5222) carried out in our laboratory, some of the animals developed symptoms of involvement of the central nervous system that might have contributed to their death The present report describes the location, extension and microscopic characteristics of the invasion of the nervous system by leukemic cells in a group of rats in which the brain and spinal cord were systematically examined

[1] Research supported by Euratom Contract No 098 72 1 BIAC (Art No BIO 1095) and the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 112 Projekt B2)

Material and Methods

Six male rats of the inbred strain BD IX [1] weighing about 200 g were transfused with 2×10^7 leukocytes obtained from the blood of a leukemic donor of the same strain. The cells had been frozen, stored under liquid nitrogen and thawed immediately before transfusion. Details of the transfer and storage procedures were given by HOELZER [2].

The acute rat leukemia L 5222 used in this experiment was chemically induced with ethylnitrosourea by IVANKOVIC and ZELLER [3] who found no evidence for virus infection by means of the Gross technique. Based on cytochemical characteristics the leukemia was classified as of immature monocytic type with a myeloid component [3].

All animals were sacrificed 6 days after transfer of the leukemic cells. A complete autopsy was performed and blocks of tissue from brain and spinal cord were fixed in formalin and imbedded in paraffin for histological studies. Shortly before sacrifice, leukocyte counts and smears were made from tail vein blood. The smears were stained with the May-Grunwald Giemsa method.

Results

Six days after intravenous injection of 2×10^7 leukemic cells all the animals had a high number of leukocytes in peripheral blood, reaching values between 265 000 and 364,000/μl. One rat was paraplegic.

The location and severity of the lesions found in the central nervous system are given in table 1. The cerebrum had no lesions in three animals and showed several small hemorrhages in three other rats. The hemorrhagic areas contained also small groups of leukemic cells (LC). The cortex was invaded in only one rat. The diencephalon showed lesions in three rats, without preference for any particular structure in that area. The cerebellum had a few small hemorrhages with LC in four rats and was normal in two animals. The meninges were diffusely infiltrated with a small number of LC in all animals.

Severe lesions were found in the spinal cord (fig. 1). All animals showed abundant LC in the epidural space spread along most of the spinal canal. Four animals had numerous hemorrhages and masses of LC, mostly in the white matter of the spinal cord. Figure 2 illustrates the location and size of the lesions in the lumbar region of one of the rats. The gray matter appeared less affected, only some areas of the dorsal column, adjacent to the hemorrhages of the white matter, were damaged.

The vertebrae were extensively infiltrated with LC. In some areas groups of LC burst through Haversian canals into the spinal canal and

Material and Methods

Six male rats of the inbred strain BD IX [1] weighing about 200 g were transfused with 2×10^7 leukocytes obtained from the blood of a leukemic donor of the same strain. The cells had been frozen, stored under liquid nitrogen and thawed immediately before transfusion. Details of the transfer and storage procedures were given by Hoellzer [2].

The acute rat leukemia L 5222 used in this experiment was chemically induced with ethylnitrosourea by Ivankovic and Zeller [3], who found no evidence for virus infection by means of the Gross technique. Based on cytochemical characteristics the leukemia was classified as of immature monocytic type with a myeloid component [3].

All animals were sacrificed 6 days after transfer of the leukemic cells. A complete autopsy was performed and blocks of tissue from brain and spinal cord were *fixed in formalin and imbedded in paraffin for* histological studies. Shortly before sacrifice leukocyte counts and smears were made from tail vein blood. The smears were stained with the May Grunwald Giemsa method.

Results

Six days after intravenous injection of 2×10^7 leukemic cells all the animals had a high number of leukocytes in peripheral blood, reaching values between 265,000 and 364,000/μl. One rat was paraplegic.

The location and severity of the lesions found in the central nervous system are given in table I. The cerebrum had no lesions in three animals and showed several small hemorrhages in three other rats. The hemorrhagic areas contained also small groups of leukemic cells (LC). The cortex was invaded in only one rat. The diencephalon showed lesions in three rats, without preference for any particular structure in that area. The cerebellum had a few small hemorrhages with LC in four rats and was normal in two animals. The meninges were diffusely infiltrated with a small number of LC in all animals.

Severe lesions were found in the spinal cord (fig. 1). All animals showed abundant LC in the epidural space spread along most of the spinal canal. Four animals had numerous hemorrhages and masses of LC, mostly in the white matter of the spinal cord. Figure 2 illustrates the location and size of the lesions in the lumbar region of one of the rats. The gray matter appeared less affected, only some areas of the dorsal column, adjacent to the hemorrhages of the white matter, were damaged.

The vertebrae were extensively infiltrated with LC. In some areas groups of LC burst through Haversian canals into the spinal canal and

Acta haemat 55 28–35 (1976)

Involvement of the Central Nervous System in Rats with Acute Leukemia L 5222[1]

W CALVO and D HOELZER

Department of Clinical Physiology, Clinical Research Center, University of Ulm, Ulm

Key Words Central nervous system hemorrhages · Leukemia · Meningeal leukemic infiltration · Rat leukemia L 5222

Abstract Young adult BD IX rats made leukemic by intravenous infusion of 2×10^7 nucleated blood cells from leukemic donors (L 5222), showed lesions in the central nervous system 6 days after transfer when their leukocyte count was between 250,000 and 350,000 cells/μl The brain was affected in only half of the animals, whereas all showed an infiltration of the meninges Furthermore, all animals had extensive lesions in the spinal cord The lesions consisted of nodules of leukemic cells and hemorrhages which were predominant in the lower half of the spinal cord and more abundant in the white than in the gray matter Paraplegia appeared in some animals shortly before death Since there is a consistent involvement of the meninges and spinal cord, the L 5222 leukemia seems to be a useful model for the study of leukemic infiltration of the central nervous system and the reaction to chemotherapy

Leukemic involvement of the central nervous system, particularly of the meninges, constitutes a neurological complication in human acute leukemia During a study of the growth characteristics of a transplantable acute leukemia in rats (L 5222) carried out in our laboratory, some of the animals developed symptoms of involvement of the central nervous system that might have contributed to their death The present report describes the location, extension and microscopic characteristics of the invasion of the nervous system by leukemic cells in a group of rats in which the brain and spinal cord were systematically examined

[1] Research supported by Euratom Contract No 088 72 1 BIAC (Art No BIO 1095) and the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 112, Projekt B2)

Fig 3 Vertebra (V) and spinal cord (SC) of rat 5 showing numerous hemorrhagic lesions in the white matter 6 days after intravenous transfusion of 2×10^7 leukocytes obtained from a donor animal of the same strain $\times 9$

spread over the dura mater along the epidural space (fig 3) Some spinal ganglia were surrounded by numerous LC The leptomeninges in the brain were diffusely infiltrated as illustrated in figure 4

Discussion

Involvement of the central nervous system in rats with leukemia L5222 is more frequent than one may suspect by observing only neurological symptoms All animals in our series had infiltration of LC in the meninges and four out of six had hemorrhages and masses of LC in several areas of the nervous system, but only one had paraplegia The main lesions were found in the lower thoracic and lumbar segments of the spinal cord Lesions in these areas are responsible for paralysis of the hind legs Extensive spread of LC in the epidural space seems to be due to invasion of LC originating in the bone marrow of the vertebrae, where they settle and proliferate shortly after transfusion [4] and communicate with the spinal canal through Haversian canals of the vertebrae

Table I Location and severity of the lesions found in the central nervous system of rats with leukaemia L 5222

Rat No	Leuko-cytes/μl	Brain			Spinal cord		
		cerebrum	cerebellum	meninges	cervical	thoracic	lumbar
1	265,800	(-)	(-)	LC (+)	LC epidural (+)	LC epidural (+)	LC epidural (+)
2	348,800	(-)	(-)	LC (+)	LC epidural (+) H and LC in white matter (+)	LC epidural (+)	LC epidural (+)
3	364,000	H and LC in dorsal cortex and corpus callosum (+)	H and LC in stratum granulosum of vermis (+)	LC (++)	LC epidural (+)	LC epidural (+)	LC epidural (+)
4	309,000	(-)	H and LC in molecular layer of vermis (+)	LC (+)	(-)	LC epidural (+)	LC epidural (+) H and LC in white matter (+)
5 paraplegic	327,000	H and LC in diencephalon (++)	H and LC in white matter of vermis (+)	LC (++)	LC epidural (+)	LC epidural (+) white matter (+) gray matter (+) H and LC (+)	LC epidural (++) H and LC in white matter (++) gray matter (+)
6	303,000	H and LC in diencephalon (+)	H and LC in white matter of vermis (+)	LC (+)	(-)	(-)	LC epidural (+) H and LC in the white matter (+)

H = Hemorrhage, LC = leukemic cell infiltraton, (++) = numerous and large, (+) = few and small, (-) = no lesions

Fig 3 Section of spinal cord (SC) and lumbar vertebra (V) The epidural space (arrow) is infiltrated with leukemic cells The white matter shows two small masses of leukemic cells (LC) and extensive hemorrhage (H) × 125

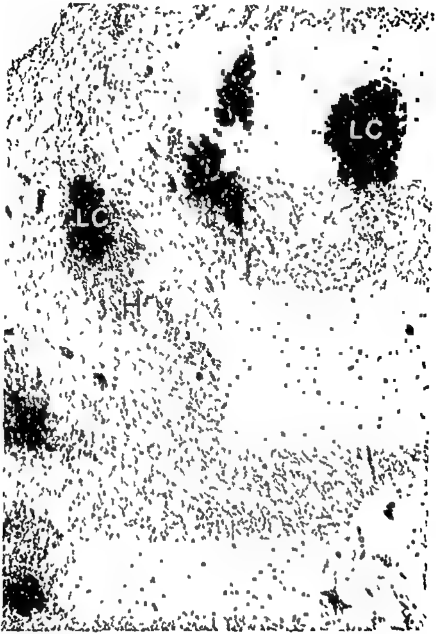


Fig 2 Section of spinal cord stained with hematoxylin and eosin. Masses of leukemic cells (LC) and associated hemorrhages (H) in the white matter of the lumbar region × 175

Fig 4 Diffuse infiltration of leukemic cells in the leptomeninges of the brain (arrow) × 500

Predominance of the lesions in the white matter may be explained by peculiarities of the vascular pattern of the spinal cord in which the nutrient vessels reach the gray matter after traversing the white matter Embolisms occasioned by the abundant large cells circulating in the blood are likely to occur more often in areas first reached by LC The cells trapped in the narrow vessels continue their proliferation and distend the vessel wall to its rupture point That such a destruction can occur has previously been demonstrated for the sinus endothelial cells of the bone marrow [5]

A contributing factor to the production of hemorrhages may be the progressive diminution of platelets in the blood of the leukemic rats In a group of animals investigated under the same conditions as described here [6] it was found that the number of platelets declined to about one tenth of normal value 6 days after transfusion of LC The bleeding may be further due to disorders of hemostasis known to develop during the course of this leukemia [7]

Involvement of the central nervous system and of the meninges in particular is a known complicating factor in human acute leukemia especial

ly in children Since involvement of the meninges is a consistent finding in the experimental L 5222 leukemia, and further since remissions can be induced in this leukemia by chemotherapy [8] it seems to be a suitable model for the study of leukemic infiltration of the central nervous system and its reaction to chemotherapy

References

1 DRUCKREY H Genotypes and phenotypes of ten inbred strains of BD rats Arzneimittel Forsch *21* 1274–1278 (1971)

2 HOELZER D Growth characteristics of a transferable acute leukemia in rats J natn Cancer Inst *5* 1321 1327 (1973)

3 IVANKOVIC S und ZELLER W J Leukamie L 5222 des Rattenstammes BD IX Eine durch Äthylnitrosoharnstoff induzierte monozytar myeloische transplantierbare Form fur zytochemische und chemotherapeutische Studien Blut *28* 288 292 (1974)

4 HOELZER D CALVO W MEYER HAMME K D and HARRISS E Cell distribution and proliferation pattern of a transferable acute leukemia in rats J natn Cancer Inst *50* 1545–1553 (1973)

5 HOELZER D HARRISS E B JAGER C HAAS R J and FLIEDNER T M Effect of the acute rat leukaemia L 5222 on bone marrow stroma cells Cancer Res *34* 1892 1897 (1974)

6 HOELZER D and HARRISS E B The failure of normal haemopoiesis in rats during the development of acute leukaemia Acta haemat. *49* 36–47 (1973)

7 RASCHE H HOELZER D DIETRICH M and KELLER A Hemostatic defects in experimental leukaemia Haemostasis *3* 46–54 (1974)

8 ZELLER W J IVANKOVIC, S., and SCHMAHL, D Chemotherapy of the transplantable acute leukemia L 5222 in rats Cancer Res *35* 1168–1174 (1975)

PD Dr D HOELZER Abteilung fur Klinische Physiologie der Universitat Ulm Parkstrasse 11 *D 79 Ulm Donau* (FRG)

Fig 4 Diffuse infiltration of leukemic cells in the leptomeninges of the brain (arrow) × 500

Predominance of the lesions in the white matter may be explained by peculiarities of the vascular pattern of the spinal cord, in which the nutrient vessels reach the gray matter after traversing the white matter Embolisms occasioned by the abundant large cells circulating in the blood are likely to occur more often in areas first reached by LC The cells trapped in the narrow vessels continue their proliferation and distend the vessel wall to its rupture point That such a destruction can occur has previously been demonstrated for the sinus endothelial cells of the bone marrow [5]

A contributing factor to the production of hemorrhages may be the progressive diminution of platelets in the blood of the leukemic rats In a group of animals investigated under the same conditions as described here [6] it was found that the number of platelets declined to about one tenth of normal value 6 days after transfusion of LC The bleeding may be further due to disorders of hemostasis known to develop during the course of this leukemia [7]

Involvement of the central nervous system and of the meninges in particular is a known complicating factor in human acute leukemia, especial-

Table I Hb Barts in 650 newborns

Amount of Hb Barts, %	Number of cases	Percent total	Percent with Hb Barts
0	621	95 54	0
1–2	21	3 23	72.4
5	7	1 08	24 2
25	1	0 15	3 4
Total	650		

determined in Cuba [1], no data are available on the incidence of α-thalassaemia We report here a study carried out to estimate the frequency of this entity

Materials and Methods

650 normal full term newborns were chosen at random from the Nursery of the E Cabrera Hospital in Habana Capillary blood was collected in heparinized tubes Haemoglobin types were identified by starch gel electrophoresis using the Tris-EDTA borate buffer of Smithies [7] at pH 8 6 Hb Barts was quantitated by cellulose acetate electrophoresis in the same buffer

In all cases showing high Hb Barts, a haematological study of the family was done, including serum iron determination

Results

Hb Barts was detected in 29 out of 650 newborns, which gives an incidence of 4 46% The amounts of Hb Barts were segregated into 3 groups showing 1–2, 5 and 25%, respectively The data are summarized in table I

Discussion

High percentages of Hb Barts are usually considered characteristic of α-thalassaemia and its levels are positively correlated with the severity of the disease [6] Moreover, the trimodal distribution of this haemoglobin found in newborns (not including the cases of hydrops fetalis, which would constitute a fourth group) allows a genetical interpretation of the α-thalassaemias, and also permits to anticipate the type of thalassaemia

Acta haemat. 55: 36–39 (1976)

α-Thalassaemia in Cuba

G. Martinez and B. Colombo

Instituto de Hematologia e Inmunologia, Habana

Key Words. α-Thalassaemia · Hb Barts · Haemoglobinopathies

Abstract. The frequency of α-thalassaemia has been determined by the analysis of Hb Barts in 650 newborns; 4.46% of them showed high levels of Hb Barts. The incidence of newborns with different levels of Hb Barts (1–2, 5 and 25% respectively) and the frequency of the α-thalassaemia genes are also given.

The α-thalassaemia syndromes are a group of hereditary microcytic anaemias of different severity, characterized by variable reductions in the rate of synthesis of the α-globin chain [11]. Genetical and clinical evidences indicate that there is more than one type of α-thalassaemia, but the genetical lesion(s) responsible for these entities is not fully understood. Recently it has been demonstrated that a deletion of the α-gene(s) produces the homozygous α-thalassaemia, which results in intrauterine or early neonatal death with a clinical picture of hydrops fetalis, associated with large amounts of haemoglobin Barts (Hb Barts) [5, 9]. On the other hand, only mild haematological changes characterize the α-thalassaemia trait [11]. Its identification is therefore extremely difficult in the adult, whereas the presence of excess γ-chains in neonates makes it possible to recognize the α-thalassaemia trait by the quantitation of Hb Barts (γ_4) during the newborn period [3]. With this method the incidence of α-thalassaemia has been determined in different countries; it has been found to be very frequent in South East Asia, where comprehensive investigations have been carried out [3, 4], and to be present also in the Mediterranean area and in African and American Negroes [8, 11]. While the frequency of the other common type of thalassaemia, the β-thalassaemia, has been

Table I Hb Barts in 650 newborns

Amount of Hb Barts, %	Number of cases	Percent total	Percent with Hb Barts
0	621	95 54	0
1–2	21	3 23	72 4
5	7	1 08	24 2
25	1	0 15	3 4
Total	650		

determined in Cuba [1], no data are available on the incidence of α-thalassaemia We report here a study carried out to estimate the frequency of this entity

Materials and Methods

650 normal full term newborns were chosen at random from the Nursery of the E Cabrera Hospital in Habana Capillary blood was collected in heparinized tubes Haemoglobin types were identified by starch gel electrophoresis using the Tris EDTA borate buffer of Smithies [7] at pH 8 6 Hb Barts was quantitated by cellulose acetate electrophoresis in the same buffer

In all cases showing high Hb Barts, a haematological study of the family was done including serum iron determination

Results

Hb Barts was detected in 29 out of 650 newborns, which gives an incidence of 4 46% The amounts of Hb Barts were segregated into 3 groups showing 1–2, 5 and 25% respectively The data are summarized in table I

Discussion

High percentages of Hb Barts are usually considered characteristic of α-thalassaemia, and its levels are positively correlated with the severity of the disease [6] Moreover, the trimodal distribution of this haemoglobin found in newborns (not including the cases of hydrops fetalis, which would constitute a fourth group) allows a genetical interpretation of the α-thalassaemias and also permits to anticipate the type of thalassaemia

which will develop in each case Thus, newborns with 25% of Hb Barts develop Hb H disease, those with 5% mild microcytic anaemia and those with 1–2% will be normal

According to the hypothesis of WASI *et al* [10], Hb H disease occurs from the interaction of two α-thalassaemia genes one is the classical severe α-thal$_1$, the other is a milder allele, α-thal$_2$ The presence of one α-thal$_1$ gene produces 5% Hb Barts in newborns, whereas the presence of one α-thal$_2$ produces levels of approximately 1–2% Another interpretation, based on the duplication of the α-chain locus, which also explains the different types of α-thalassaemia, has been proposed by KATTAMIS and LEHMANN [2] According to this model, the inactivity of one, 2, 3 or of all the 4 α-genes is responsible for the various types of the disease

Under either hypothesis a family study must be done to assess the incidence of the different thalassaemic genes Thus, only one parent of patients with Hb H disease (carrying 25% Hb Barts at birth) shows the α-thalassaemia trait, both parents of individuals with 1–2% Hb Barts at birth are normal, whereas two types of inheritance are expected in families of individuals with 5% Hb Barts either only one parent shows the α-thalassaemia trait, or both are silent carriers Family studies of the 29 newborns found in this screening gave the following pattern

1 Group with 1–2% Hb Barts In each family of the 21 cases, only one parent showed mild morphological alterations, the other being completely normal

2 Group with 5% Hb Barts In this group of 7 newborns, two types of inheritance were found (a) In two families only one parent was affected by the classical α-thalassaemia trait, with morphological alterations and microcythaemia, while the other was completely normal (b) In 5 families both parents showed only mild morphological alterations

3 Group with 25% Hb Barts The only case showing 25% Hb Barts had one parent with alterations typical of the α-thalassaemia trait, the other showing only mild morphological alterations

Based on these findings, the frequencies of the α-thalassaemia genes were calculated assuming the existence of two allelic genes, α-thal$_1$ and α-thal$_2$ [6] It turned out that the frequency of the α-thal$_1$ (severe) gene was 0 0023 and that of the α-thal$_2$ (mild) allele was 0 0240 The number of individuals with 1–2, 5 and 25% of Hb Barts found in the study was the expected according to the Hardy-Weinberg law

The Cuban population derives mainly from two groups Caucasoids from Spain and Negroes from Africa, the contribution of Mongoloids al-

though very small, cannot be neglected, especially in a study on the frequency of α thalassaemia The genetic admixture between these groups has produced an almost continuous variation of the most conspicuous aspects related to the racial pertinency, which makes it very difficult to assess the racial origin However, when a classification based on the colour of the skin thickness of the lips hair, etc is applied to the families reported above, it appears that the three races have introduced the α-thalassaemia genes in Cuba but it is impossible to determine their relative contribution

References

1 De la Torre E Hernandez, P Martinez G Svarch E and Colombo B β Thalassaemia in Cuba Haematologia (in press)

2 Kattamis C and Lehmann H The genetical interpretation of haemoglobin H disease Hum Hered *20* 156–164 (1970)

3 Na Nakorn S and Wasi P Alpha thalassaemia in Northern Thailand Am J hum Genet *22* 645–651 (1970)

4 Na Nakorn S Wasi P and Suingduvrong A Hemoglobin H disease in Thailand clinical and hematological studies of 138 cases Israel J med Scis *1* 762 764 (1965)

5 Ottolenghi S Lanyon W G Paul J Williamson R Weatherall D J Clegg J B Pritchard J Pootrakul S and Boon W H The severe form of α thalassaemia is caused by a haemoglobin gene deletion Nature Lond *251* 389 392 (1974)

6 Pootrakul S Wasi P and Na Nakorn S Studies on haemoglobin Bart s in Thailand the incidence and the mechanism of occurrence in cord blood Ann hum Genet *31* 149 166 (1967)

7 Smithies, O An improved procedure for starch gel electrophoresis further variations in the serum proteins of normal individuals Biochem J *71* 585–587 (1959)

8 Schwartz E and Atwater J α Thalassaemia in the American Negro J clin Invest *51* 412–418 (1972)

9 Taylor J M Dozy A Kan Y W Varmus H E Lie Injo L E Ganesan J and Todd D Genetic lesion in homozygous α thalassaemia Nature Lond *251* 392 394 (1974)

10 Wasi P Na Nakorn S and Suingdumrong A Haemoglobin H disease in Thailand a genetical study Nature Lond *204* 907 908 (1964)

11 Weatherall D J and Clegg J B The thalassaemia syndromes (Blackwell Oxford 1972)

Dr B Colombo Instituto de Hematologia e Inmunologia Hospital F Cabrera Apartado 8070 Altahabana *La Habana 8* (Cuba)

though very small, cannot be neglected, especially in a study on the frequency of α-thalassaemia The genetic admixture between these groups has produced an almost continuous variation of the most conspicuous aspects related to the racial pertinency, which makes it very difficult to assess the racial origin. However, when a classification based on the colour of the skin, thickness of the lips, hair, etc. is applied to the families reported above, it appears that the three races have introduced the α-thalassaemia genes in Cuba, but it is impossible to determine their relative contribution

References

1 De la Torre E, Hernandez, P, Martinez, G, Svarch, E, and Colombo B β-Thalassaemia in Cuba Haematologia (in press)

2 Kattamis, C and Lehmann, H The genetical interpretation of haemoglobin H disease Hum Hered *20* 156–164 (1970)

3 Na Nakorn, S and Wasi, P Alpha thalassaemia in Northern Thailand Am J hum Genet *22* 645–651 (1970)

4 Na Nakorn, S, Wasi, P, and Suingdumrong, A Hemoglobin H disease in Thailand clinical and hematological studies of 138 cases Israel J med Scis *1* 762–764 (1965)

5 Ottolenghi, S, Lanyon, W G, Paul, J, Williamson, R Weatherall, D J, Clegg, J B, Pritchard, J, Pootrakul, S., and Boon, W H The severe form of α thalassaemia is caused by a haemoglobin gene deletion Nature, Lond *251* 389–392 (1974)

6 Pootrakul, S, Wasi, P, and Na Nakorn S Studies on haemoglobin Bart's in Thailand the incidence and the mechanism of occurrence in cord blood Ann hum Genet *31* 149–166 (1967)

7 Smithies, O An improved procedure for starch gel electrophoresis further variations in the serum proteins of normal individuals Biochem J *71* 585–587 (1959)

8 Schwartz, L and Atwater J α Thalassaemia in the American Negro J clin Invest *51* 412–418 (1972)

9 Taylor J M Dozy A Kan Y W Varmus, H E, Lie Injo, L E, Ganesan J and Todd D Genetic lesion in homozygous α thalassaemia. Nature, Lond *251* 392 394 (1974)

10 Wasi P Na Nakorn S and Suingdumrong, A Haemoglobin H disease in Thailand a genetical study Nature Lond *204* 907–908 (1964)

11 Weatherall, D J and Clegg J B The thalassaemia syndromes (Blackwell Oxford 1972)

Dr B Colombo Instituto de Hematologia e Inmunologia, Hospital E. Cabrera, Apartado 6070, Altahabana, *La Habana 8* (Cuba)

which will develop in each case. Thus, newborns with 25% of Hb Barts develop Hb H disease, those with 5% mild microcytic anaemia and those with 1–2% will be normal.

According to the hypothesis of WASI *et al.* [10], Hb H disease occurs from the interaction of two α-thalassaemia genes: one is the classical severe α-thal$_1$, the other is a milder allele, α-thal$_2$. The presence of one α-thal$_1$ gene produces 5% Hb Barts in newborns, whereas the presence of one α-thal$_2$ produces levels of approximately 1–2%. Another interpretation, based on the duplication of the α-chain locus, which also explains the different types of α-thalassaemia, has been proposed by KATTAMIS and LEHMANN [2]. According to this model, the inactivity of one, 2, 3 or of all the 4 α-genes is responsible for the various types of the disease.

Under either hypothesis a family study must be done to assess the incidence of the different thalassaemic genes. Thus, only one parent of patients with Hb H disease (carrying 25% Hb Barts at birth) shows the α-thalassaemia trait, both parents of individuals with 1–2% Hb Barts at birth are normal, whereas two types of inheritance are expected in families of individuals with 5% Hb Barts: either only one parent shows the α-thalassaemia trait, or both are silent carriers. Family studies of the 29 newborns found in this screening gave the following pattern:

1. Group with 1–2% Hb Barts. In each family of the 21 cases, only one parent showed mild morphological alterations, the other being completely normal.

2. Group with 5% Hb Barts. In this group of 7 newborns, two types of inheritance were found. (a) In two families only one parent was affected by the classical α-thalassaemia trait, with morphological alterations and microcythaemia, while the other was completely normal. (b) In 5 families both parents showed only mild morphological alterations.

3. Group with 25% Hb Barts. The only case showing 25% Hb Barts had one parent with alterations typical of the α-thalassaemia trait, the other showing only mild morphological alterations.

Based on these findings, the frequencies of the α-thalassaemia genes were calculated assuming the existence of two allelic genes, α-thal$_1$ and α-thal$_2$ [6]. It turned out that the frequency of the α-thal$_1$ (severe) gene was 0.0023 and that of the α-thal$_2$ (mild) allele was 0.0240. The number of individuals with 1–2, 5 and 25% of Hb Barts found in the study was the expected according to the Hardy-Weinberg law.

The Cuban population derives mainly from two groups, Caucasoids from Spain and Negroes from Africa, the contribution of Mongoloids, al-

which will develop in each case Thus, newborns with 25% of Hb Barts develop Hb H disease, those with 5% mild microcytic anaemia and those with 1–2% will be normal

According to the hypothesis of WASI *et al* [10], Hb H disease occurs from the interaction of two α-thalassaemia genes one is the classical severe α-thal$_1$, the other is a milder allele, α-thal$_2$ The presence of one α-thal$_1$ gene produces 5% Hb Barts in newborns, whereas the presence of one α-thal$_2$ produces levels of approximately 1–2% Another interpretation, based on the duplication of the α-chain locus, which also explains the different types of α-thalassaemia, has been proposed by KATTAMIS and LEHMANN [2] According to this model, the inactivity of one, 2, 3 or of all the 4 α-genes is responsible for the various types of the disease

Under either hypothesis a family study must be done to assess the incidence of the different thalassaemic genes Thus, only one parent of patients with Hb H disease (carrying 25% Hb Barts at birth) shows the α-thalassaemia trait, both parents of individuals with 1–2% Hb Barts at birth are normal, whereas two types of inheritance are expected in families of individuals with 5% Hb Barts either only one parent shows the α-thalassaemia trait, or both are silent carriers Family studies of the 29 newborns found in this screening gave the following pattern

1 Group with 1–2% Hb Barts In each family of the 21 cases, only one parent showed mild morphological alterations the other being completely normal

2 Group with 5% Hb Barts In this group of 7 newborns, two types of inheritance were found (a) In two families only one parent was affected by the classical α-thalassaemia trait, with morphological alterations and microcythaemia, while the other was completely normal (b) In 5 families both parents showed only mild morphological alterations

3 Group with 25% Hb Barts The only case showing 25% Hb Barts had one parent with alterations typical of the α-thalassaemia trait, the other showing only mild morphological alterations

Based on these findings, the frequencies of the α-thalassaemia genes were calculated assuming the existence of two allelic genes, α-thal$_1$ and α-thal$_2$ [6] It turned out that the frequency of the α-thal$_1$ (severe) gene was 0 0023 and that of the α-thal$_2$ (mild) allele was 0 0240 The number of individuals with 1–2, 5 and 25% of Hb Barts found in the study was the expected according to the Hardy-Weinberg law

The Cuban population derives mainly from two groups, Caucasoids from Spain and Negroes from Africa, the contribution of Mongoloids, al-

though very small, cannot be neglected especially in a study on the frequency of α-thalassaemia The genetic admixture between these groups has produced an almost continuous variation of the most conspicuous aspects related to the racial pertinency, which makes it very difficult to assess the racial origin However, when a classification based on the colour of the skin, thickness of the lips hair, etc is applied to the families reported above, it appears that the three races have introduced the α thalassaemia genes in Cuba, but it is impossible to determine their relative contribution

References

1 De la Torre E Hernandez P Martinez G Svarch E and Colombo B β Thalassaemia in Cuba Haematologia (in press)

2 Kattamis C and Lehmann H The genetical interpretation of haemoglobin H disease Hum Hered *20* 156–164 (1970)

3 Na Nakorn S and Wasi P Alpha thalassaemia in Northern Thailand Am J hum Genet *22* 645–651 (1970)

4 Na Nakorn S Wasi P and Suingduvrong A Hemoglobin H disease in Thailand clinical and hematological studies of 138 cases Israel J med Scis *1* 762–764 (1965)

5 Ottolenghi S Lanyon W G Paul J Williamson R Weatherall D J Clegg J B Pritchard J Pootrakul S and Boon W H The severe form of α thalassaemia is caused by a haemoglobin gene deletion Nature Lond *251* 389 392 (1974)

6 Pootrakul S Wasi P and Na Nakorn S Studies on haemoglobin Bart's in Thailand the incidence and the mechanism of occurrence in cord blood Ann hum Genet *31* 149 166 (1967)

7 Smithies O An improved procedure for starch gel electrophoresis further variations in the serum proteins of normal individuals Biochem J *71* 585–587 (1959)

8 Schwartz E and Atwater J α Thalassaemia in the American Negro J clin Invest *51* 412–418 (1972)

9 Taylor J M Dozy A Kan Y W Varmus H E Lie Injo L E Ganesan J and Todd D Genetic lesion in homozygous α thalassaemia Nature Lond *251* 392 394 (1974)

10 Wasi P Na Nakorn S and Suingdumrong A Haemoglobin H disease in Thailand a genetical study Nature Lond *204* 907 908 (1964)

11 Weatherall D J and Clegg J B The thalassaemia syndromes (Blackwell Oxford 1972)

Dr B Colombo Instituto de Hematologia e Inmunologia Hospital E. Cabrera Apartado 8070 Altahabana *La Habana 8* (Cuba)

Acta haemat 55 40–47 (1976)

Study of a Large Anglo-Saxon Family with β-Thalassaemia Trait

EVA RAIK, ELIZABETH POWELL and SUSAN GORDON

Haematology Department, Kanematsu Memorial Institute, Sydney Hospital, Sydney

Key Words Anglo Saxon family · Genetics · Heterogeneity of β thalassaemia · Inheritance of β thalassaemia · Thalassaemia

Abstract Study of a large Anglo Saxon family with β thalassaemia trait revealed evidence of consanguinity, moreover both branches of the family shared a Spanish ancestor The manifestations of the disorder were varied in severity and yet the degree of severity appeared to breed true within any individual part of the family Our explanation for the inheritance pattern observed in the family was to postulate the existence of two non allelic genes influencing the rate of β chain synthesis

At one time β-thalassaemia was not considered a likely cause of refractory hypochromic anaemia in patients of other than Mediterranean origin Evidence of this disease in Britain is now well documented [1, 2, 5] We have investigated 91 members of a very large apparently Anglo-Saxon family This family is of particular interest for two reasons Firstly, the family is able to trace its history back five generations to a Spanish sea captain This evidence of Mediterranean ancestry is similar to that found in one British family described by CALLENDER *et al* [1] who had a Spanish ancestor three generations earlier Secondly, despite a consanguinous marriage, there is no evidence of homozygosity, only the heterozygous state, varying in degree of severity, is evident

Materials and Methods

Our proposita was referred to us by her local doctor for confirmation of a suspected β thalassaemia trait As the patient was of Anglo-Saxon origin we decid

Table I Haematological results of affected family members shown in figure 1

Case No	Age	Sex	Hb, g%	MCH, pg	MCV, μm^3	Abnormal red cell morphology	HbA_2 %	HbF, %
V 1	61	F	13 9	30 1	82	−	5 0	1.2 (1 1)
V 2	66	M	13 1	NT	NT	+	8 0	6 0 (3 0)
VI 1 (proposita)	37	F	11.2	21 1	61	+ +	8 3	7 1
VI 2	34	F	13 0	NT	NT	−	5 2	1 5 (2 0)
VI 3	35	F	10 8	NT	NT	+ +	7 0	6 0 (2 0)
VI-4	31	F	12 5	31 8	87	−	7 5	1 1 (0 8)
VI-6	22	F	9 4	NT	NT	+ +	11 0	7 0 (2 0)
VII 1	12	M	10 5	NT	NT	+ +	8 5	3 0 (2 0)
VII-6	9	F	10 3	NT	NT	+ +	8 0	6 0 (3 0)
VII 9	6	M	11 7	NT	NT	−	6 0	5 0 (3 0)

HbF control figures for aged blood samples are shown in parentheses

ed to test all the members of the family who could be traced The family proved to be both interested and co-operative The mother of the proposita was able to provide a great deal of detail about the family tree and it transpires that she and her husband are distant cousins having a great grandmother in common

Altogether 91 members representing both branches of the family were investigated Full investigations were not always possible because many members of the family resided in country areas of Australia and the blood samples were often some days in transit Whenever feasible the following investigations were performed by standard procedures : full haematological profile from the Coulter Counter Model S, which we have found to be a sensitive index of hypochromia and microcytosis Unfortunately this instrument was not available for testing the initial family members ; reticulocyte count examination of a stained blood film haemoglobin electrophoresis on cellulose acetate (cellogel) using Tris-EDTA borate barbitone buffer at pH 8 6

HbA_2 levels were assessed by dissolving the appropriate bands after staining the strips with Ponceau S and reading the optical density of the resultant solutions in a colorimeter The normal range for this laboratory is 1 7–4 7% with a mean of 3 2% .

Fetal haemoglobin (HbF) levels were determined by the alkali denaturation method of BETKE as described by LEHMANN and HUNTSMAN [6] In this laboratory the normal level on fresh blood is <0 8% , however in the survey samples were frequently some days in transit and occasionally in poor condition on arrival Whenever possible controls of similar age were used on such occasions and the

Acta haemat 55 40–47 (1976)

Study of a Large Anglo-Saxon Family with β-Thalassaemia Trait

EVA RAIK, ELIZABETH POWELL and SUSAN GORDON

Haematology Department Kanematsu Memorial Institute, Sydney Hospital Sydney

Key Words Anglo Saxon family Genetics Heterogeneity of β thalassaemia Inheritance of β thalassaemia Thalassaemia

Abstract Study of a large Anglo Saxon family with β thalassaemia trait revealed evidence of consanguinity moreover both branches of the family shared a Spanish ancestor The manifestations of the disorder were varied in severity and yet the degree of severity appeared to breed true within any individual part of the family Our explanation for the inheritance pattern observed in the family was to postulate the existence of two non allelic genes influencing the rate of β chain synthesis

At one time β-thalassaemia was not considered a likely cause of refractory hypochromic anaemia in patients of other than Mediterranean origin Evidence of this disease in Britain is now well documented [1, 2, 5] We have investigated 91 members of a very large apparently Anglo-Saxon family This family is of particular interest for two reasons Firstly, the family is able to trace it s history back five generations to a Spanish sea captain This evidence of Mediterranean ancestry is similar to that found in one British family described by CALLENDER *et al* [1] who had a Spanish ancestor three generations earlier Secondly, despite a consanguinous marriage, there is no evidence of homozygosity, only the heterozygous state, varying in degree of severity, is evident

Materials and Methods

Our *proposita* was referred to us by her local doctor for confirmation of a suspected β thalassaemia trait As the patient was of Anglo Saxon origin we decid

Table I Haematological results of affected family members shown in figure 1

Case No	Age	Sex	Hb, g %	MCH, pg	MCV, μm^3	Abnormal red cell morphology	HbA_2 %	HbF, %
V 1	61	F	13 9	30 1	[illegible]	−	5 0	1 2 (1 1)
V 2	66	M	13 1	NT	NT	+	8 0	6 0 (3 0)
VI 1 (proposita)	37	F	11 2	21 1	61	+ +	8 3	7 1
VI 2	34	F	13 0	NT	NT	−	5 2	1 5 (2.0)
VI 3	35	F	10 8	NT	NT	+ +	7 0	6 0 (2 0)
VI-4	31	F	12.5	31 8	87	−	7 5	1 1 (0 8)
VI-6	22	F	9 4	NT	NT	+ +	11 0	7 0 (2 0)
VII 1	12	M	10 5	NT	NT	+ +	8 5	3 0 (2 0)
VII-6	9	F	10 3	NT	NT	+ +	8 0	6 0 (3 0)
VII 9	6	M	11 7	NT	NT	−	6 0	5 0 (3 0)

HbF control figures for aged blood samples are shown in parentheses

ed to test all the members of the family who could be traced The family proved to be both interested and co-operative The mother of the proposita was able to provide a great deal of detail about the family tree and it transpires that she and her husband are distant cousins having a great grandmother in common

Altogether 91 members representing both branches of the family were investigated Full investigations were not always possible because many members of the family resided in country areas of Australia and the blood samples were often some days in transit Whenever feasible the following investigations were performed by standard procedures full haematological profile from the Coulter Counter Model S which we have found to be a sensitive index of hypochromia and microcytosis Unfortunately this instrument was not available for testing the initial family members reticulocyte count examination of a stained blood film haemoglobin electrophoresis on cellulose acetate (cellogel) using Tris EDTA borate barbitone buffer at pH 8 6

HbA_2 levels were assessed by dissolving the appropriate bands after staining the strips with Ponceau S and reading the optical density of the resultant solutions in a colorimeter The normal range for this laboratory is 1 7–4 7% with a mean of 3 2%

Fetal haemoglobin (HbF) levels were determined by the alkali denaturation method of BETKE as described by LEHMANN and HUNTSMAN [6] In this laboratory, the normal level on fresh blood is <0 8%, however in the survey samples were frequently some days in transit and occasionally in poor condition on arrival Whenever possible controls of similar age were used on such occasions and the

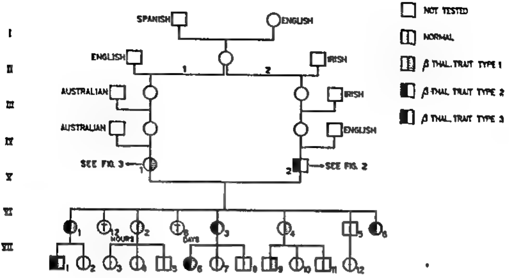


Fig 1 Abbreviated pedigree of the proposita (VI-1) illustrating the consanguinity and the Spanish ancestry

Table II Haematological results on affected family members shown in figure 2 (except V 2)

Case No	Age	Sex	Hb, g%	MCH, pg	MCV, μm^3	Abnormal red cell morphology	HbA_2, %	HbF, %
V-7	59	M	10 8	21 6	69	+ +	5 5	1 0 (0 8)
VI-13	22	F	12 5	30 7	84	–	5 7	1 1 (0 8)
VI-17	adult	F	11 1	20 7	66	+	8 8	3 0 (1 0)
VII-16	34	M	11 7	21 3	64	+ +	8 4	2 7 (1 0)
VII-18	25	F	12 9	29 7	86	–	6 5	0 8
VII-25	9	F	12 2	20 0	57	+ +	5 0	2 8 (1 1)
VIII-1	6	F	9 8	20 3	61	+ +	8 1	3 0 (1 0)
VIII-2	9	F	10 7	20 2	61	+ +	7 5	3 1 (1 0)
VIII-3	2	M	10 3	19 8	60	+ +	10 4	3 1 (1 0)

VI 8, VI-18 tested at another hospital and diagnosed HbF control figures for aged blood samples are shown in parentheses

results interpreted accordingly In tables I, II, the appropriate control figure for any aged sample is shown in parentheses

Serum iron levels were not determined because many of the family were from country areas and suitable sera were difficult to obtain Moreover some were currently receiving iron therapy

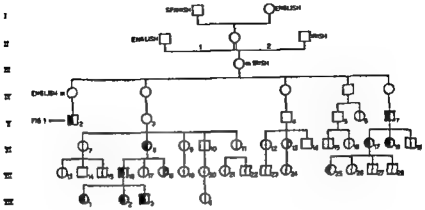


Fig 2 Paternal branch of the family For explanation of symbols, see fig 1

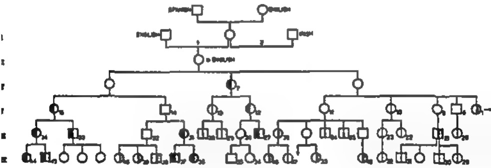


Fig 3 Maternal branch of the family For explanation of symbols, see fig 1

Results

For convenience, the family tree has been greatly simplified and divided into three sections Figure 1 shows the pedigree of the proposita and her immediate family, figure 2 depicts her father's family and figure 3 the maternal branch of the family The haematological results are similarly divided and summarised in tables I–III, except for VI–8 and VI–18 who were investigated and diagnosed at other hospitals

Thirty-nine family members of the 91 investigated showed evidence of β-thalassaemia trait in some form Thirteen members (from both branches) showed an elevated HbA_2 level with no other detectable ab-

Table III Haematological results of affected family members shown in figure 3 (excep V-1)

Case No	Age	Sex	Hb, g%	MCH, pg	MCV, μm^3	Abnormal red cell morphology	HbA_2, %	HbF, %
IV-7	>80	F	12 0	21 4	67	+	7 1	2 4 (1 0)
V-12	adult	F	11 9	22 8	74	+	5 9	3 0 (0 8)
V-15	adult	F	11 0	21 3	66	+	8 4	4 4
VI-21	54	M	16 5	32 4	96	−	6 6	0 9
VI 27	child	M	12 7	19 8	64	+	6 2	1 0 (0 8)
VI 31	*40*	*F*	*11 6*	*19 9*	*58*	+	*10 3*	*1 2 (0 8)*
VI-33	37	M	13 2	22 7	75	+	6 7	2 0 (0 8)
VI-34	adult	F	10 2	21 0	63	+ +	7 5	1 7 (1 0)
VI 35	49	F	14 1	31 5	89	−	7 0	1 0 (0 8)
VII-33	adult	F	13 4	31 5	90	−	6 0	0 9
VII-36	child	F	11 5	NT	NT	+	8 0	3 7
VII 37	*13*	*M*	*11 5*	*19 3*	*56*	+	*10 8*	*1 7 (0 8)*
VII-38	child	F	12 9	29 9	82	−	6 3	0 9
VII 39	child	M	12 7	29 7	82	−	5 6	0 7
VII-40	child	F	13 1	29 3	82	−	5 3	1 0
VII-43	6	M	10 5	19 0	57	+ +	8 6	1 7 (1 0)
VII-44	4	F	11 2	20 2	60	+ +	8 8	3 0 (1 0)
VII-45	23	F	13 0	NT	NT	−	6 4	0 8
VII 46	23	F	12 7	28 3	79	−	5 4	1 4 (1 0)

HbF control figures for aged blood samples are shown in parentheses

normality Of the remaining β-thalassaemia traits in the family, all had elevations of both HbA_2 and HbF levels and 15 were also anaemic In the case of V–7, the anaemia and marked red cell changes may have been accentuated by the co-existence of multiple myeloma

Discussion

It is now generally accepted that the abnormality in the thalassaemias lies not in the structure of the globin chains but in the quantity of the particular chain produced The actual mechanism whereby chain production is decreased is as yet unknown Recently, it has been shown [3

Table IV Haematological results of family Z. and family S

Case No	Age	Sex	Hb, g %	MCH, pg	MCV, μm^3	Abnormal red cell morphology	HbA_2, %	HbF, %
Family Z								
Y Z., proposita	40	F	8 9	18 7	67	+ + +	[illegible]	6 5
Husband R Z.	43	M	14 6	29 3	[illegible]	–	3 0	0 5
Brother J M	50	M	14 5	NT	NT	–	1 7	1 0
Sister N B		F	12 2	25 2	72	–	5 4	1 0
Daughter T Z.	21	F	12.6	23 1	67	+	6 6	0 6
Daughter C. Z.[1]	18	F	12.2	21 9	67	+	4 8	0 4
Daughter S Z.[1]	18	F	12.3	23 7	70	+	5 5	0 8
Family S								
C S., proposita	30	F	8 3	19 3	59	+ + +	8 4	1 8
Husband V S	30	M	14 1	NT	NT	–	2 5	0 8
Daughter K S	6	F	14 1	24 4	70	+	6 7	1 1
Son J S	2	M	11 5	23 0	66	+	10	1 7

[1] Non identical twins

4] that the actual amount of βmRNA in β-thalassaemia is lower than normal This could result from a β structural gene altered to produce βmRNA less efficiently, or a separate 'thalassaemia' gene exerting an inhibitory effect on the β-structural gene

β-Thalassaemia trait manifests with varying degrees of severity, however, it is usually fairly constant in its manifestations in any one family It is apparent from the results that these degrees of severity exist throughout both sides of this family

To facilitate the discussion it has been necessary to allot an arbitrary classification for the patterns of β thalassaemia trait seen in this family For convenience we designated them as follows Type 1 normal with the exception of an increase in HbA_2, type 2 deficient haemoglobinisation and abnormal red cell morphology, increased levels of HbA_2 and HbF, type 3 symptomatic anaemia, deficient haemoglobinisation with more marked changes in red cell morphology, increased levels of HbA_2 and HbF It may appear that by attempting such a classification we are drawing too fine a line of distinction between the variations of β thalassaemia trait We must stress therefore that the blood films were exam-

Heterozygous and Homozygous Factor XI Defect in a Consanguineous Family

Effect of Age on Heterozygous Expression

G I C Ingram and C R Rizza

Haematology Department St Thomas' Hospital and Medical School London and Oxford Haemophilia Centre, Churchill Hospital, Oxford

Key Words Age effect on factor XI Bleeding disorders Coagulation Factor XI defect

Abstract A woman with symptoms of prolonged post traumatic bleeding was found to have a severe defect of factor XI, and was thought to be homozygously affected Both her parents who were first cousins, and all her three children though asymptomatic, showed a moderate defect and appeared to be heterozygous Both her parents factor XI levels were about twice those in her children and this was thought to be an age effect

Various clotting factors may rise or fall with age [1–3], factor XI is reported [1] to rise with age in women but to decrease in men We report a consanguineous Jewish family transmitting a factor XI defect, the proband appears to be homozygously affected and heterozygotes can be identified in both the previous and subsequent generations The older heterozygotes, a man and a woman, have much higher factor XI levels than the three younger heterozygotes suggesting that the levels in both the grand parents have risen with age

Laboratory Methods

Blood samples were anticoagulated with trisodium citrate dihydrate solution For the preliminary tests 9 vol of blood were mixed with 1 vol 3.2% solution and for the factor XI assays 49 vol of blood were mixed with 1 vol 20% solution Samples were handled and tested throughout in plastic equipment

The one stage prothrombin time test was performed with Manchester Comparative Reagent (phenolized suspension of human brain) and 25 mM calcium chloride

solution, and the result expressed as the ratio of the patient's time to the control time

The partial thromboplastin time with kaolin involved an initial preincubation of equal volumes of the patient's citrated plasma and a suspension of light kaolin 5 mg/ml in Tris buffer 50 mM (pH 7.4 at room temperature) for 10 min, the reaction mixture was then completed by adding volumes of Thrombofax (Ortho) and 25 mM calcium-chloride solution. The upper limit of normal is taken as 6 sec longer than the control [4]

Contact product was prepared by Pérez Requejo's modification [5] of Nossel's method [6] As a screening test for contact factor defects, the partial thromboplastin time test was repeated, adding a volume of contact product after the calcium-chloride solution instead of the initial preincubation with kaolin If a prolonged result with kaolin was normalised by contact product, the plasma sample was considered to be defective in contact factor activity [7]

In factor XI assays for family members III3, III6, IV1, IV2, V1, V2 and V3 (fig 1) natural factor XI deficient plasma was available as substrate For members IV4, V4, V5 and V6, an artificial factor XI deficient substrate [8] was used The assays were performed as partial thromboplastin times with kaolin on aliquots of the substrates to which had been added three serial dilutions of the test plasma or, as a standard, three dilutions of a pool of three fresh normal plasmas. The factor XI activities were calculated from the results by the standard methods for parallel line bioassays [9]

All tests were performed at 37 °C.

Case Report and Family Details

The family tree is shown in figure 1 No details are available for generations I and II In generation III, members 3 and 6 are the grandparents of the present study Neither has unequivocal symptoms of abnormal bleeding although III6 bled 'severly' after an operation for prolapse, but the bleeding was controlled by packing and she was not transfused

The proband, IV2, bled for 'some days' and for 2 days after two dental extractions She bled in the night following an operation for prolapse and she was transfused 3.5 litres blood A haematoma formed after a breast operation and the wound bled intermittently for 1 month She has had menorrhagia only since the birth of her children Results of her preliminary clotting tests are given in table I Her husband, IV1, who is her second cousin, has no history of abnormal bleeding Her sister, IV4, suffered a post partum haemorrhage after the birth of her first child for which she received a transfusion of plasma but whole blood was not given, otherwise, she has had 'numerous' dental extractions with a normal haemostatic response None of the six children in generation V have any history of defective haemostasis, V5 has had teeth extracted and V6 tonsillectomy without abnormal bleeding

Factor XI levels, ABO blood groups and dates of birth are given in figure 1 The figure also shows that III3, IV2 and V1 suffer from Raynaud's phenomenon, proba

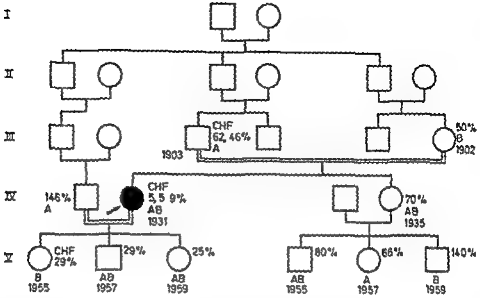


Fig 1 Family tree Factor XI%, A and B blood groups, year of birth and the incidence of 'cold hands and feet' (CHF Raynaud's phenomenon) are shown Arrow indicates proband who had symptoms of abnormal bleeding

Table I Preliminary clotting tests in proband

Test	Result	
Prothrombin time ratio	0 93	
Partial thromboplastin time with kaolin, sec		
Control	36 9	
Patient	48 4	
Difference from control		+11 5
Control + patient 50 50	37.2	
Difference from control		+0 3
Partial thromboplastin time with contact product, sec		
Control	40 1	
Patient	38 4	
Difference from control		−1 7

The patient's partial thromboplastin time with kaolin was prolonged, but was corrected by equal mixture with normal plasma, substituting contact product for kaolin activation also corrected the patient's abnormality

bly all since childhood, fingers and toes blanch in the cold but there have been no complications

Operative cover in the proband The proband subsequently underwent an anterior and posterior perineal repair Preoperative cover was provided by approximately 1 litre of fresh noncontacted plasma, and her factor XI level rose from 6 to 37%. Similar doses of frozen uncontacted plasma were given after 11 and 23 h, after the second her level rose from 29 to 36% Tranexamic acid, 1 g every 6 h was also given by mouth from the preoperative day for 7 days, and for a further week at 0.5 g every 6 h, in addition, a supplementary intravenous dose of 3 g ε aminocaproic acid was given preoperatively Bleeding at operation was thought to be somewhat excessive but haemostasis was secured postoperative bleeding was not abnormal

Discussion

The distribution of A and B blood groups confirm the family relationships shown The simplest interpretation of the distribution of factor XI levels and the occurrence of bleeding symptoms is that proband IV2 is homozygous for the defective gene, and that both her parents and all her children are heterozygotes It is then necessary to explain why her parents' levels of factor XI are approximately twice the levels in her children, and it seems likely that this is an age effect Her parents are some 55 years older than her children, and an average increase of approximately 0 5% per annum for both the grandparents would account for the difference, a value within the range for factors VII and IX found by others [2, 3] This is contrary to the evidence of HAMILTON *et al* [1] who reported a rise in females but a fall in males with age among normal subjects

The haemostatic responses of the heterozygotes, and of the proband at her various operations, suggest that at least for this particular mutation, 25–30% of factor XI activity seems to be adequate for normal haemostasis, though 5–10% does not.

Acknowledgements We are grateful to Dr J EASTON for referring the proband to us, and to Miss M E CORK and Miss J PHILLIPS for carrying out the factor XI assays We are grateful to Dr J H RENWICK who kindly discussed the genetics of this family with us

References

1 HAMILTON P J, ALLARDYCE, M, OGSTON D, DAWSON A. A., and DOUGLAS, A S. The effect of age upon the coagulation system J clin. Path. 27 980 (1974)

2 Brozović, M, Stirling, Y, Harricks, C., North, W R S, and Meade, T W Factor VII in an industrial population Br J Haemat 28 381 (1974)
3 Dodds, W J, Moynihan, A C., Benson, R E, and Hall, C A The value of age- and sex matched controls for coagulation studies Br J Haemat 29 305 (1975)
4 Matchett, M O and Ingram, G I C Partial thromboplastin test with kaolin Normal range and modifications for the diagnosis of haemophilia and Christmas disease J clin Path 18 465 (1965)
5 Pérez-Requejo, J L and Ingram, G I C The preparation of 'contact product as a reagent for coagulation tests J clin Path 28 596 (1975)
6 Nossel, H L The contact phase of blood coagulation, p 124 (Blackwell Oxford 1964)
7 Ingram, G I C, Knights S F, Arocha Piñango C L, Shepperd, J P, Pérez Requejo, J L, and Mills, D K Simple screening tests for the diagnosis of isolated clotting factor defects. J clin Path 28 524 (1975)
8 Blewitt, R E A new method for the assay of factor XI in plasma and the preparation and use of a new artificial substrate J clin Path 28 332 (1975)
9 Ingram, G I C Blood coagulation factor VIII genetics physiological control and bioassay, in Sobotka and Stewart Advances in clinical chemistry, vol 8 pp 227-235 (Academic Press, New York 1965)

Prof G I C Ingram Department of Haematology, St Thomas' Hospital and Medical School, *London SE1 7EH* (England)

Acta haemat 55 53–59 (1976)

Circulating IgG Antibodies against Factors IX and VIII in Multiple Sclerosis[1]

J L. MOAKE, C. J KENT, L. D META and L C. WRIGHT

Hematology Research Laboratories University of Texas Medical School Houston Tex., and the University of Miami School of Medicine Miami Fla

Key Words Antibodies against clotting factors · Anticoagulants · Blood coagulation · Factor VIII inhibitor · Factor IX inhibitor · Multiple sclerosis

Abstract A 64-year-old man with multiple sclerosis developed a circulating anticoagulant Three immunoglobulin fractions (I = IgG II = IgG+IgA III = IgM) were separated from patient serum by 33-percent ammonium sulfate precipitation and DEAE 52 cellulose column chromatography Fractions I (IgG) and II (IgG+IgA) had factor VIII inhibitory activity on incubation Fraction I (IgG) possessed in addition immediate anti IX activity which did not require incubation Absorption studies characterized the VIII inhibitors as IgG $\varkappa$ in fraction I and IgG $\varkappa$+IgG λ in fraction II Immediately reactive IgG anti IX antibodies associated with kinetically distinct IgG anti VIII antibodies have not been described previously

Circulating inhibitors of factor VIII [1] have appeared in previously transfused hemophilia A patients [2], in normal women following parturition, in elderly individuals, and in patients with immunological disorders [3] These inhibitors often have been IgG antibodies [1] although IgA and IgM VIII inhibitors have been described [4, 5] The IgG antibodies frequently have reacted predominantly with anti-$\varkappa$ light-chain antisera [1 6] and in several patients have been subclassified as IgG-4 or IgG 3 [7, 8] Incubation of normal plasma with VIII inhibitors often has resulted in further loss of VIII activity [1, 6, 9] Most acquired inhibitors of factor IX have been detected in previously transfused hemophilia B patients [1] These IgG antibodies (IgG-4 λ in 1 patient [10]) have produced immediate

[1] Supported in part by the Howard Hughes Medical Foundation and by NIH Grant No 1 S01 RR05745-01

interference with the IX activity of normal plasma without further decrease after incubation [1, 11]

We have studied a nonhemophilic patient with IgG antibodies inhibiting the activities of both factor IX and factor VIII

Case Report

Patient S S, a 64 year-old white male, developed diplopia and ataxia He was found to have generalized hyperreflexia with clonus bilateral Babinski and Hoffman signs, bilateral optic pallor, poor memory and calculating ability, and labile affect There were diffuse electroencephalographic abnormalities Skull films and brain scan were normal γ globulins were increased in a polyclonal electrophoretic pattern in serum and cerebrospinal fluid Additional studies included normal hemoglobin white cell count and differential platelet count, prothrombin time (PT) and activated partial thromboplastin time (APTT) Erythrocyte sedimentation rate was 115 mm/h Antinuclear antibodies and rheumatoid factor were not present. Serum chemical profile was normal, as were serum B_{12} and folate levels

Three years later, he developed hematuria and extensive ecchymoses There was no previous bleeding history and no family history of hemorrhagic disorder He had never received a blood transfusion On admission the hemoglobin was 9.2 g%, reticulocytes 0 8%, white cell count 6 200 (normal differential) and platelets 382,000/μl Red cells were microcytic and hypochromic. Table I summarizes initial coagulation studies Additionally, the following were normal Ivy bleeding time (3 min, 15 sec), tourniquet test clot retraction, fibrin degradation products (<10 μg/ml) thrombin time (patient 20 sec/control 20 sec), and fibrinogen (202 mg%) Hemolytic assays of serum complement components [12] were normal serum CH_{50} values were 256 000 units/ml for C4 and 19,200 units/ml for C3

Materials and Methods

Plasma recalcification time (PRT) assays [13] were performed by adding 0 2 ml of 0 02 M $CaCl_2$ to 0 2 ml plasma For crossed PRT determinations patient and control plasmas were mixed to 0 2 ml final volumes and tested immediately Inhibitor concentration was estimated by diluting patient plasma in Tris buffered saline (Tris HCl 0 05 M + NaCl 0 15 M pH 7 4) to a final volume of 0.2 ml prior to the addition of 0 2 ml fresh normal plasma and 0 2 ml 0 02 M $CaCl_2$

APTT and PT determinations were done using standard techniques

Assays for coagulation factors [13] were done using single factor-deficient plasmas obtained from Dade Reagents Factors XII XI IX, and VIII were assayed by methods based on the one stage APTT and assays for factors VII, V and prothrombin were done by a one stage PT method Factor X was assayed by both APTT and PT methods

Separation and purification of serum immunoglobulins were done by 33 per cent ammonium sulfate precipitation [14] and DEAE 52-cellulose (Reeve Angle

Clifton, N J) anion exchange chromatography [15] using a continuous gradient KH_2PO_4/K_2HPO_4 (pH 8 0) buffer Column fractions I, II and III were dialyzed against Tris NaCl and adjusted to concentrations of 7 mg/ml Immunoglobulins were identified by immunodiffusion [16] against antibodies to IgG, IgA, IgM IgD, IgE, $\varkappa$, and λ light-chains (Meloy Laboratories, Springfield, Va.) Anticoagulant activity in fractions I, II, and III was determined by mixing 0 05 ml with 0 05 ml of fresh normal plasma and performing APTT determinations and specific factor assays immediately and following incubation for 1 h at 37 °C.

Antisera to IgG IgA, IgM, $\varkappa$, and λ light-chains were absorbed for 1 h at 23 °C with 100 mg/ml $BaSO_4$ centrifuged, and incubated for 30 min at 56 °C to destroy coagulation factor activity in the antisera [10, 11] The column fractions were combined with absorbed antisera in ratios ranging from 1 1 to 1 8 (fraction antisera) and incubated at 37 °C for 1 h and at 4 °C for 16 h. Each antiserum treated column fraction (0 1 ml total volume) was added to 0 1 ml fresh normal plasma, and factor assays were performed immediately and following incubation at 37 °C for 1 h Control factor levels were determined by (1) combining 0 1 ml fresh normal plasma with 0 1 ml Tris NaCl (to determine dilutional effect on factor levels), and (2) incubating each column fraction with Tris NaCl at 37 and 4 °C in ratios equivalent to those with antisera prior to performing factor assays (to determine inhibitory activity in diluted fractions) Each column fraction antiserum combination was tested in immunodiffusion plates versus pooled normal human serum Precipitin lines indicated antiserum in excess of column fraction immunoglobulins

Results

Initial coagulation studies Patient S S had an inhibitor directed against the intrinsic coagulation pathway (table I) Factor X activity in patient plasma was undetectable by APTT method and 80% by PT assay Patient plasma required a 50- to 100-fold dilution to eliminate the anticoagulant effect.

Characterization of anticoagulant Three serum fractions containing immunoglobulins were obtained by DEAE-52 cellulose chromatography IgG (fractions I and II), IgA (fraction II), and IgM (fraction III) IgD and IgE were not detected Only fractions I and II had anticoagulant activity The mixture of fraction I with fresh normal plasma resulted in an immediate 50-percent decrease in factor IX activity not accentuated by incubation In contrast, anti-VIII activity was not detected immediately, however, after incubation of fraction I or fraction II with fresh normal plasma VIII activity was reduced by >90% There were no inhibitory effects on the activities of factors XII, XI, or X by either fraction

The anti VIII effect of fraction I was 80% reversed by absorption with IgG antiserum and completely reversed by absorption with $\varkappa$-anti

Table I Initial coagulation studies

	Plasma recalcification time (seconds)	Activated partial thromboplastin time (seconds)	Prothrombin time (seconds)
Patient plasma	>400	97	11
Normal plasma	100	35	11

Patient plasma	100%	75%	50%	25%	10%	0
Normal plasma	0	25%	50%	75%	90%	100%
Plasma recalcification time (seconds)[1]	>400	253	218	202	195	100

	Activated partial thromboplastin time method[2]					Prothrombin Time Method[3]			
Factor	*XII*	*XI*	*VIII*	*IX*	*X*	*X*	*VII*	*V*	*II*
Patient plasma activity (%)	1 7	<5	3 1	3 5	0	80	85	88	103

[1] Final volume 0 2 ml
[2] Patient plasma 0 1 ml + ellagic acid phospholipid 0 1 ml + 0 02 M $CaCl_2$ 0 1 ml
[3] *Patient plasma 0 1 ml + thromboplastin − $CaCl_2$ [illegible]2 ml*

serum Partial reversal of fraction II VIII-inhibitory activity followed absorption with IgG (40%), $\varkappa$ (60%), and λ (55%) antisera Ratios of fraction to antisera were 1 8 At this dilution antisera to $\varkappa$- and λ-light-chains were in excess with fractions I and II, IgA antiserum was in excess with fraction II, and IgG antiserum was in excess with fraction II Anti-VIII activity was not consistently present at fraction I dilutions exceeding 1 8 It was not possible to characterize the immediate anti-IX activity in fraction I since dilutions in excess of threefold did not possess effects which could be reversed by fraction I antisera ratios less than 1 3

Discussion

Patient S [illegible] developed a severe hemorrhagic disorder associated with multiple sclerosis An anticoagulant with immediate inhibitory activity

against the intrinsic coagulation pathway was detected by initial studies (prolonged PRT and APTT, normal PT, abnormal crossed PRT), and very low levels of factors XII, XI, VIII, IX, and X were found However, normal levels of X, as well as VII, V, and prothrombin, were obtained in assays using a one stage PT method The normal factor X by PT assay and undetectable X activity by APTT assay suggested that the anticoagulant was directed against the intrinsic activator of factor X, either activated factor IX (IXa) or the complex of phospholipid, Ca^{++}, factor VIII, and IXa [17]

Anion exchange chromatography separated two serum immunoglobulin fractions with anticoagulant properties Fraction I (IgG) had immediate inhibitory effects on factor IX activity, and fractions I (IgG) and II (IgG+IgA) inhibited factor VIII activity following incubation The IgG VIII inhibitor in fraction I was exclusively of the $\varkappa$ light-chain type Because the anti IX effect of fraction I IgG disappeared upon 1 3 dilution it was not possible to determine with antiserum absorption experiments whether anti IX antiobdy was monoclonal or polyclonal IgG antibodies of both $\varkappa$- and λ light-chain types were responsible for the anti-VIII effects of fraction II Fraction II IgG antibodies may have been predominantly of IgG-4 subclass, since IgG-4 (which is more positively charged than IgG 1, IgG 2, and IgG 3 [18, 19]) is eluted with IgA from anion exchange columns Acquired VIII inhibitors in other patients have been characterized as IgG-4 antibodies [7, 8]

IgG-4 does not activate complement via the C1 pathway, and IgG-2 does so poorly when compared with IgG 1 and IgG 3 [20] The normal serum C4 and C3 levels implied that excessive complement activation through the C1 or C3 bypass (properdin) pathways [21] did not result from IgG reactions with factor IX or factor VIII Thus, the IgG VIII inhibitor in fraction II may have been IgG-4, and the anti IX and anti VIII antibodies in fraction I may have been IgG 2

Factor VIII and IX assays by one stage APTT methods involve the generation of phospholipid, Ca^{++}, VIII IXa complexes following the addition of test plasma dilutions to VIII- or IX-deficient plasma If the fraction I IgG had been directed against the VIII IXa complex than immediate inhibition of normal plasma VIII activity and further decrease in IX activity with incubation should have been found However, the kinetic behavior of the fraction I IgG inhibitors of IX and VIII were distinct Apparently different IgG antibodies in fraction I inhibited the activities of factors IX and VIII

It is unlikely that the IgG antibodies reacted with phospholipid components of the complex involving VIII, IXa, and Ca^{++}, since neither fraction I nor fraction II inhibited X activity (which requires formation of Xa V-phospholipid Ca^{++} complexes). Phospholipid may be the plasma component against which the lupus anticoagulant [1] is directed. Immediately reactive IgG and/or IgM antibodies [22, 23] interfere with coagulation in some patients with systemic lupus erythematosus and other immunological disorders [1]. The lupus anticoagulant is usually associated with prolonged PT, and bleeding has infrequently occurred in the absence of thrombocytopenia or reduced prothrombin levels [1].

Anticoagulants against factor IX occur following transfusion in some patients with Christmas disease. Acquired factor IX deficiency has been reported in systemic lupus erythematosus [24], rheumatic fever [25], and myeloid metaplasia [25]. Patient S. S. is unique in that both anti-IX and anti-VIII IgG antibodies were present in association with multiple sclerosis.

Acknowledgements. Dr. DUANE R. SCHULTZ kindly performed serum C4 and C3 measurements.

References

1. FEINSTEIN, D. I. and RAPAPORT, S. I.: Acquired inhibitors of blood coagulation. Prog. Hemostasis Thromb. *1:* 75–95 (1972).
2. STRAUSS, H. S.: Acquired circulating anticoagulants in hemophilia A. New Engl. J. Med. *281:* 866–873 (1969).
3. MARGOLIUS, A.; JACKSON, D. P., and RATNOFF, O. D.: Circulating anticoagulants: a study of 40 cases and a review of the literature. Medicine *40:* 145–202 (1961).
4. GLUECK, H. I. and HONG, R.: A circulating anticoagulant in γ1A multiple myeloma: its modification by penicillin. J. clin. Invest. *44:* 1866–1881 (1965).
5. MCKELVEY, E. M. and KWAAN, H. C.: An IgM circulating anticoagulant with factor VIII inhibitory activity. Ann. intern. Med. *77:* 571–575 (1972).
6. SHAPIRO, S. S.: The immunologic character of acquired inhibitors of antihemophilic globulin (factor VIII) and the kinetics of their interaction with factor VIII. J. clin. Invest. *46:* 147–156 (1967).
7. ANDERSEN, B. R. and TERRY, W. D.: Gamma G4-globulin antibody causing inhibition of clotting factor VIII. Nature, Lond. *217:* 174–175 (1968).
8. ROBBOY, S. J.; LEWIS, E. J.; SCHUR, P. H., and COLMAN, R. W.: Circulating anticoagulants to factor VIII. Am. J. Med. *49:* 742–752 (1970).
9. GREEN, D.: Spontaneous inhibitors of factor VIII. Br. J. Haemat. *15:* 57–75 (1968).

10 PIKE, I M, YOUNT W J, PURITZ, E. M., and ROBERTS H R Immunochemical characterization of a monoclonal γG4 λ human activity to factor IX. Blood *40* 1–10 (1972)
11 ROBERTS H R, GROSS G P, WEBSTER W P, DEJANOV, I I., and PENICK, G D Acquired inhibitors of plasma factor IX A study of their induction properties, and neutralization Am J med Sci *251* 43–50 (1966)
12 NELSON [illegible] A Proteins of the complement system and their biologic activity, in PEETERS Protides of the biologic fluids vol 15, pp 385–399 (Elsevier Amsterdam 1968)
13 BOWIE, E J W THOMPSON J H, DIDISHEIM P., and OWEN C A in Mayo Clinic Laboratory manual of hemostasis (Saunders Philadelphia 1971)
14 STELLOS [illegible] Salt fractionation in WEIS Handbook of experimental immunolo[illegible]gy pp 3–9 (Davis, Philadelphia 1967)
15 FAHEY J L, and TERRY, D W Ion exchange chromatography and gel filtration in WEIS Handbook of experimental immunology (Davis Philadelphia 1967)
16 OUCHTERLONY [illegible] Immunodiffusion and immunoelectrophoresis in WEIS Handbook of experimental immunology pp 655–706 (Davis Philadelphia 1967)
17 NEMERSON Y and PITLICK, F A The tissue factor pathway of blood coagulation Prog Hemostasis Thromb *1* 1–37 (1972)
18 TURNER M W, BENNICH, H H., and NATVIG J B Pepsin digestion of human G myeloma protein of different subclasses Clin exp Immunol 7 603–640 (1970)
19 GERGELY J MEDGYESI G A WANG A C., and FUDENBERG H H IgG myeloma subclass typing by tryptic digestion of whole sera Immunochem 9 589–592 (1972)
20 ISHIZAKA, T ISHIZAKA K, SALMON S and FUDENBERG H Biologic activities of aggregated gamma globulin VIII Aggregated immunoglobulins of different classes J Immun 99 82–91 (1967)
21 GOTZE, [illegible] and MULLER EBERHARD H J The C3 activator system an alternate pathway of complement activation J exp Med *134* 905–1095 (1971)
22 YIN E. T and GASTON L. W Purification and kinetic studies on a circulating anticoagulant in a suspected case of lupus erythematosus. Thromb Diath. haemorrh *14* 88–115 (1965)
23 LECHNER K A new type of coagulation inhibitor Thromb Diath. haemorrh. *32* 482–499 (1969)
24 CASTRO O FARBER L. R., and CLYNE, L. P Circulating anticoagulants against factor IX and XI in systemic lupus erythematosus Ann intern. Med 77 543–548 (1972)
25 OZSOYLU S and OZER F L. Acquired factor IX deficiency Acta haemat *50* 305–314 (1973)

JOEL L. MOAKE MD Associate Professor [illegible] Medicine Director Division [illegible] Hematology University of Texas Medical School 6400 West Cullen, *Houston TX 77025* (USA)

Acta haemat. 55 60–64 (1976)

Infectious Mononucleosis in a Patient with *Hodgkin's Disease*

M M A C Langenhuysen

Division of Hematology, Department of Medicine, University Hospital, Groningen

Key Words Epstein Barr virus Hodgkin's disease Infectious mononucleosis

Abstract The case is presented of a patient, treated for Hodgkin's disease, who contracted infectious mononucleosis more than 3 years later While Epstein Barr virus has been considered a possible etiologic factor in Hodgkin's disease, the sequence of events reported in this case has to be interpreted as evidence against a causal relationship between the virus and Hodgkin's disease in this patient

The prior occurrence of infectious mononucleosis reported in several cases of Hodgkin's disease [8, 18] might point to a relationship between both diseases In an extensive investigation, 29 cases have been found, with an interval from the diagnosis of infectious mononucleosis to that of Hodgkin's disease of less than 1 year in eight [8] In three cases the infectious mononucleosis related lymphadenopathy persisted and merged into the subsequent Hodgkin's disease In a cohort study of cancer following infectious mononucleosis in Connecticut [2], reports of 4,529 cases of infectious mononucleosis could be identified through existing records and matched against a register of cancer cases because both are reportable diseases in that state Relative risks were found elevated for certain forms of cancer, the largest risk being that for malignant lymphoma (7 cases observed, 1 9 expected, relative risk 3 7) In five cases infectious mononucleosis preceded Hodgkin's disease by 3–11 years In another cohort study [15] no significant differences in mortality from Hodgkin's disease were found in a group of 2,437 veterans with infectious mononucleosis diagnosed in 1944 and a similar number of controls Sternberg-Reed cells, until recently considered pathognomonic for Hodgkin's disease, have been observed in lymph

nodes of patients with infectious mononucleosis [12] and in recurrent Burkitt lymphoma [19]

There is widespread agreement now that infectious mononucleosis is brought about by Epstein-Barr virus (EBV) in individuals who were seronegative for EBV before [reviews 9, 14] Although a strong serologic and virologic relationship exists between the virus and Burkitt lymphoma and nasopharyngeal carcinoma [9, 14] an etiologic role for EBV in either malignancy cannot be considered established Several investigators have found a serologic relationship between EBV and Hodgkin's disease Both anti-EBV virus capsid antigen (VCA) antibodies, determined by an indirect immunofluorescence technique on fixed EBV-carrying lymphoblastoid cell lines [5, 7, 10, 11], and antibodies, reacting with specific cell surface antigens [7] were significantly elevated in patients as opposed to controls No difference in titers of antibody to the closely related cytomegalovirus (CMV) could be demonstrated between patient and control groups [11] These antibodies were assayed by the relatively insensitive complement fixation technique We therefore measured antibody titers to both EBV-VCA and CMV in sera of untreated patients with Hodgkin's disease and in age- and sex matched controls, using the sensitive indirect immunofluorescence technique [10] Both antibody levels were found to be significantly elevated in patients as opposed to controls These results cast some doubt on a specific role of EBV in Hodgkin's disease The finding of EBV-seronegative patients [10, 11] may be interpreted as evidence against an etiologic role for EBV at least in these cases of Hodgkin's disease

The occurrence of infectious mononucleosis in a patient, treated more than 3 years before with curative radiotherapy for Hodgkin's disease, is reported in this paper It is considered clear evidence of a lack of causal relationship between EBV and Hodgkin's disease in this patient

Methods

Antibody titers against EBV-VCA and EBV-associated nuclear antigen (EBNA) were titrated in pre-illness, acute phase and reconvalescent sera of the patient.

The indirect immunofluorescence technique for the detection of anti-EBV VCA antibodies was performed as described before [10]. The indirect immunofluorescence test for anti EBNA antibodies was a modification of procedures described before [4, 17]. The substrate was the P3HR1 cell line The cells were brought onto slides, air-dried and fixed in acetone at − 20°C for 10 min Patient sera were inactivated at 56°C for 30 min and applied to the cells *in twofold dilutions. The preparations* were then incubated for 30 min at room temperature, washed and incubated for 30 min at room temperature with

a complement-containing serum without detectable antibodies to EBV, diluted 1:6. After further washings FITC-conjugated anti human $\beta 1C/\beta 1A$ globulin (Hyland Laboratories), diluted 1:30 was applied. After incubation and washings, the slides were dried and mounted with coverslips and glycerol phosphate-buffered saline (PBS). Dilutions were made in PBS. Known positive and negative sera were included. Human diploid embryonic fibroblasts were used as negative substrate. A positive reaction was characterized by a finely granular staining of the nuclei of almost all P3HR1 cells.

Case Report

The patient was a 17 year-old boy at his first admission in 1971. He then underwent diagnostic thoracotomy for a large right sided mediastinal mass. Pathologic examination revealed the nodular sclerosis type of Hodgkin's disease. He was staged CSIAPSI[illegible] [1]. Examination of peripheral blood leukocyte concentrates and spleen histology led to the assignment into the localized form [3]. Head and neck, mediastinal and axillary glands were irradiated in a single so-called mantle field (Cobald, 4000 rad in 38 days). He remained healthy until November 1974, more than 3 years after the irradiation. He was readmitted with a history of fever up to 39 °C, headache and sore throat of 12 days duration. Physical examination revealed a generalized lymphadenopathy. Hemoglobin 15.9 g/100 ml, hematocrit 47%, white blood cell count 28 800/µl with 79% mononuclear cells among which were 71% atypical lymphocytes, platelet count 271,000/µl. Serum alkaline phosphatase 30 Bessey units (normal 0.8–3.5), SGOT 143 Wroblewski units (normal 5–40), SGPT 194 Wroblewski units (normal 5–35), bilirubin <1 mg/100 ml. Monosticon test positive, heterophil titer $\geq$ 1:512 after guinea pig kidney absorption. Anti EBV antibody titers are summarized in table I. A seroconversion for both anti EBV VCA and anti EBNA antibodies could be demonstrated in the acute phase serum as opposed to pre-illness sera. The diagnosis was infectious mononucleosis. All symptoms and signs faded off in a few weeks. The patient is healthy and without signs of Hodgkin's disease at the time of writing (February 1975).

Discussion

The diagnosis of infectious mononucleosis in this patient is justified by the clinical and laboratory findings, including a high heterophil titer after guinea pig kidney absorption. It is confirmed by the seroconversion concerning anti-EBV VCA and anti-EBNA antibodies. Obviously, this patient contracted a primary infection with EBV more than 3 years after curative treatment of Hodgkin's disease. Therefore EBV cannot be considered an etiological factor in the origin of Hodgkin's disease in this patient. It is tempting to speculate on an etiologic role for herpesviruses in lymphoma. At least four herpesviruses have been held responsible for lymphoma in animals: Marek's virus in chickens [16], *Herpesvirus saimiri* and *Herpesvirus*

Table I Serological data before, during and after infectious mononucleosis in a patient with Hodgkin's disease

Days before (−) and after (+) onset of symptoms	Anti EBV antibody titers		Heterophil titer after guinea pig kidney absorption
	anti EBV VCA	anti EBNA	
− 1138	neg.		
− 955	neg.	neg.	
+ 15	1,280	4	> 512
+ 79	1,280	2	

neg = Anti EBV VCA titer < 10, anti EBNA titer < 2.

ateles in monkeys [13] and *Herpesvirus sylvilagus* in rabbits [6] As Hodgkin's disease may well represent an etiologically heterogeneous group, it cannot be excluded that EBV could trigger the disease process in individual cases

References

1 Carbone P P, Kaplan H S, Musshoff, K, Smithers, D W., and Tubiana, M Report of the committee on Hodgkin's disease staging classification Cancer Res. *31* 1860–1861 (1971)

2 Connelly, R. R and Christine, B W A cohort study of cancer following infectious mononucleosis Cancer Res. *34* 1172 1178 (1974)

3 Halie, M R, Eibergen R., and Nieweg, H O Observations on abnormal cells in the peripheral blood and spleen in Hodgkin's disease Brit med. J *ii* 609–611 (1972)

4 Henle, G, Henle, W., and Horwitz, C. A Antibodies to Epstein-Barr virus-associated nuclear antigen in infectious mononucleosis J infect Dis *130* 231–239 (1974)

5 Hesse, J, Andersson, E., Levine, P H, Ebbesen, P, Halberg, P., and Reisher, J J Antibodies to Epstein Barr virus and cellular immunity in Hodgkin's disease and chronic lymphatic leukemia Int J Cancer *11* 237–243 (1973)

6 Hinze, H C. and Chipman, P J Role of herpesviruses in malignant lymphoma in rabbits Fed Proc. Fed Am. Socs exp Biol *31* 1639–1642 (1972)

7 Johansson B, Klein, C., Henle, W., and Henle, G Epstein Barr virus (EBV)-associated antibody patterns in malignant lymphoma and leukemia. I Hodgkin's disease Int J Cancer *6* 450–462 (1970)

8 Kaplan, H S Hodgkin's disease, pp 32–39 (Harvard University Press, Cambridge 1972)

9 Klein, G The Epstein-Barr virus (EBV) in Kaplan The herpesviruses, pp 521–555 (Academic Press, New York 1973)

10 LANGENHUYSEN, M M A C., CAZEMIER T, HOUWEN B, BROUWERS, T M, HALIE M R, THE, T H, and NIEWEG H O Antibodies to Epstein Barr virus cytomegalo virus, and Australia antigen in Hodgkin's disease Cancer *34* 262–267 (1974)
11 LEVINE, P H, ABLASHI, D V, BERARD, C. W, CARBONE, P P, WAGGONER, D E., and MALAN, L. Elevated antibody titers to Epstein Barr virus in Hodgkin's disease Cancer *27* 416–421 (1971)
12 LUKES, R. J, TINDLE, B H, and PARKER, J W Reed-Sternberg like cells in infectious mononucleosis Lancet *ii* 1003–1004 (1969)
13 MELÉNDEZ, L V, HUNT, R D, DANIEL, M D, FRASER, C. E. O, BARAHONA, H H, KING, N W, and GARCÍA, F G *Herpesviruses saimiri* and *ateles* Their role in malignant lymphomas of monkeys Fed Proc. Fed Am. Socs exp Biol *31* 1643–1650 (1972)
14 MILLER, G The oncogenicity of Epstein Barr virus J infect. Dis *130* 187–205 (1974).
15 MILLER, R. W and BEEBE, G W Infectious mononucleosis and the empirical risk of cancer J nat Cancer Inst *50* 315–321 (1973)
16 PURCHASE, H G Role of herpesviruses in Marek s disease, a malignant lymphoma of chickens Fed Proc. Fed Am Socs exp Biol *31* 1634–1638 (1972)
17 REEDMAN, B M and KLEIN G Cellular localization of an Epstein Barr virus (EBV)-associated complement fixing antigen in producer and non producer lymphoblastoid cell lines Int J Cancer *11* 499–520 (1973)
18 SMITHERS, D W Hodgkin s disease II Brit med J *ii* 337–341 (1967)
19 WRIGHT, D W Reed Sternberg like cells in recurrent Burkitt lymphomas Lancet *i* 1052 (1970)

Dr M M A C. LANGENHUYSEN, Division of Hematology Department of Medicine University Hospital 59 Oostersingel, *Groningen* (The Netherlands)

Acta haemat. 55: 65–72 (1976)

Types of Leukemia in Chronic Benzene Poisoning. A Study in Thirty-Four Patients[1]

MUZAFFER AKSOY, ŞAKIR ERDEM and GÜNÇAĞ DINÇOL

Section of Hematology, Internal Clinic of Istanbul Medical School, Çapa, Istanbul

Key Words. Benzene poisoning · Leukemia · Preleukemia · Shoe workers

Abstract. The distribution of various types of leukemia due to chronic exposure to benzene is described in a series comprising 34 cases. The incidence of leukemia among 31 shoe workers was 13.5/100 000. Acute myeloblastic leukemia was the most frequent type, followed by preleukemia, acute erythroleukemia and acute lymphoblastic leukemia. The extreme rarity of chronic myeloid leukemia was a noteworthy finding. The differences and similarities between the distribution of various types of leukemia in different series of patients with chronic exposure to benzene and ionizing radiation are discussed.

The etiology of leukemia is still obscure. Despite this fact, the role of ionizing radiation and benzene in the development of leukemia is well-known [3–5, 8, 9, 11–17, 19]. During the period from 1967 to 1973, we collected 26 shoe-workers chronically exposed to benzene with acute leukemia in Istanbul and showed that the incidence of leukemia among them was 13/100,000 which is a marked and statistically significant increase over that of leukemia in the general population [3]. In addition, in 1974 we have observed 8 new individuals with leukemia associated with chronic benzene poisoning. The purpose of this paper is to report them and compare the types of leukemia in individuals chronically exposed to benzene and those exposed to ionizing radiations.

[1] This study was supported by a grant (TAG 328) from the Scientific and Technical Research Council of Turkey.

10 [illegible]
... antigen in Hodgkin's disease Cancer *34* 262–267 (1974)
11 LEVINE, P H, ABLASHI, D V, BERARD, C W, CARBONE, P P, WAGGONER, D E, and MALAN, L Elevated antibody titers to Epstein Barr virus in Hodgkin's disease Cancer *27* 416–421 (1971)
12 LUKES, R J, TINDLE, B H, and PARKER, J W Reed Sternberg like cells in infectious mononucleosis Lancet *ii* 1003–1004 (1969)
13 MELÉNDEZ, L V, HUNT, R D, DANIEL, M D, FRASER, C E O, BARAHONA, H H, KING, N W, and GARCÍA F G *Herpesviruses saimiri* and *ateles* Their role in malignant lymphomas of monkeys Fed Proc Fed Am Socs exp Biol *31* 1643–1650 (1972)
14 MILLER, G The oncogenicity of Epstein Barr virus J infect Dis *130* 187–205 (1974)
15 MILLER, R W and BEEBE, G W Infectious mononucleosis and the empirical risk of cancer J nat Cancer Inst *50* 315–321 (1973)
16 PURCHASE, H G Role of herpesviruses in Marek's disease, a malignant lymphoma of chickens Fed Proc Fed Am Socs exp Biol *31* 1634–1638 (1972)
17 REEDMAN, B M and KLEIN, G Cellular localization of an Epstein Barr virus (EBV)-associated complement fixing antigen in producer and non producer lymphoblastoid cell lines Int J Cancer *11* 499–520 (1973)
18 SMITHERS, D W Hodgkin's disease II Brit med J *ii* 337–341 (1967)
19 WRIGHT, D W Reed Sternberg like cells in recurrent Burkitt lymphomas Lancet *i* 1052 (1970)

Dr M M A C LANGENHUYSEN, Division of Hematology, Department of Medicine, University Hospital, 59 Oostersingel, *Groningen* (The Netherlands)

Acta haemat. 55 65–72 (1976)

Types of Leukemia in Chronic Benzene Poisoning. A Study in Thirty-Four Patients[1]

Muzaffer Aksoy, Sakir Erdem and Günçağ Dinçol

Section of Hematology, Internal Clinic of Istanbul Medical School, Çapa, Istanbul

Key Words Benzene poisoning Leukemia Preleukemia Shoe workers

Abstract The distribution of various types of leukemia due to chronic exposure to benzene is described in a series comprising 34 cases The incidence of leukemia among 31 shoe workers was 13.5/100,000 Acute myeloblastic leukemia was the most frequent type followed by preleukemia, acute erythroleukemia and acute lymphoblastic leukemia The extreme rarity of chronic myeloid leukemia was a noteworthy finding The differences and similarities between the distribution of various types of leukemia in different series of patients with chronic exposure to benzene and ionizing radiation are discussed.

The etiology of leukemia is still obscure Despite this fact, the role of ionizing radiation and benzene in the development of leukemia is well-known [3–5, 8, 9, 11–17, 19] During the period from 1967 to 1973, we collected 26 shoe-workers chronically exposed to benzene with acute leukemia in Istanbul and showed that the incidence of leukemia among them was 13/100,000 which is a marked and statistically significant increase over that of leukemia in the general population [3] In addition, in 1974 we have observed 8 new individuals with leukemia associated with chronic benzene poisoning The purpose of this paper is to report them and compare the types of leukemia in individuals chronically exposed to benzene and those exposed to ionizing radiations

[1] This study was supported by a grant (TAG/328) from the Scientific and Technical Research Council of Turkey

Material and Methods

According to the official records there are 28,500 workers involved in the shoe slipper and handbag industry in Istanbul [3] The working conditions, including the mode of benzene exposure are described elsewhere [3, 6, 7] In Istanbul, during the period from 1967 to 1974, 33 leukemic individuals chronically exposed to benzene[1] were found With the exception of 2 (1 furniture worker, 1 working in a printing shop), all were shoe workers Twenty six of these patients were described elsewhere [3–5] In 1974, 8 new leukemic patients with chronic exposure to benzene were studied in the Internal Clinic of Istanbul Medical School The concentrations of benzene in their working environment measured by a Drager-Spürgerät Multi Gas Detector Mod 21 23, Lubek, were recorded to reach 210 or, rarely, 640 ppm when benzene containing adhesives were used

Results

Some clinical and hematological findings and the duration of exposure are given in tables I and II

Types of leukemia The comparison of the distribution of the types of leukemia found in these 34 individuals chronically exposed to benzene and those of 50 non-exposed leukemic patients is given in table III Similarly, the ages of these non-exposed leukemic individuals who have been studied in our clinic during the last 1½ years varied between 16 and 60 years with a mean of 37 7 years According to our results, acute myeloblastic leukemia seems to be the most frequent variety of this malignancy following exposure to benzene Then comes preleukemia, erythroleukemia and acute lymphoblastic leukemia The criteria for the diagnosis of preleukemia were similar to those of WINTROBE *et al* [20] (1) the presence of refractory anemia, mostly hypochromic or pancytopenia, (2) the percentages of myeloblasts in the bone marrow within the normal range or slightly increased, and (3) the presence of a few blast cells, mostly myeloblasts, in the peripheral blood film

Pancytopenic period A preceding pancytopenic period was present in 23 5% of all cases It was noted in eight patients, three with acute myeloblastic leukemia, three with preleukemia, one with acute monocytic leukemia and one with acute erythroleukemia Preceding pancytopenic period was particularly present in 50% of preleukemic patients The interval between the onset of leukemia and that of pancytopenic period varied from 6 months to 6 years It was the shortest in case 5 and the longest in

[1] One of the 34 leukemic patients was exposed to benzene outside of Istanbul

Table I Clinical, hematological and bone marrow findings in 8 new leukemic individuals through exposure to benzene

	Cases 1	2	3	4	5	6	7
Age, years	36	53	18	41	21	45	28
Duration of exposure, years	7	20	1–2	4–12	5	8	1½
Pancytopenic period	+	–	–	+	+	–	–
Type of leukemia	AEL	AEL	ALL	PL	PL	CML	AML
Hematological findings							
RBC × $10^6/\mu l$	1.98	1.41	2.60	1.96	2.00	4.00	2.70
Hb, g%	3.9	3.6	7.3	5.4	4	10.1	8
WBC/μl	2,500	5,600	39,000	2,800	5,600	331,000	13,400
Reticulocytes, %	2.8	1	1	1.5	1	1	2.8
Platelets, × $10^3/\mu l$	10.0	15.0	10.0	11.5	10.0	300	90.0
Hematocrit, %	16	14	22	[illegible]	14	41	25
Myeloblasts, %	0	0	0	6	10	1	0
Lymphoblasts, %	0	0	60	0	0	0	0
Myelocytes, %	0	0	0	0	0	25	0
Monocytes, %	3	2	1	0	2	0	91
Nucleated red blood cells/100 WBC	11	3	0	0	4	1	1
Bone marrow findings							
Myeloblasts, %	12	40		5	2[1]		4
Monocytes, %	0	0		0	0		66
Megaloblasts, %	12	0		0	1		0
Myeloid series, %	35	48		[illegible]	46		13
Erythroid series, %	55	48.5		2	33		14

AEL = Acute erythroleukemia, ALL = acute lymphoblastic leukemia, PL = preleukemia, CML = c myeloid leukemia, AML = acute monocytic leukemia, + = present, – = absent.

[1] These findings were delivered by the bone marrow puncture performed in the preceding pancyt period. Two other bone marrow punctures performed during the leukemic phase yielded no mater

case 4, six months and six years respectively. Usually, the clinical and hematological findings of pancytopenia improved considerably or even disappeared completely. But despite this improvement, the findings of leukemia developed later, following a period of variable duration.

Incidence of leukemia. During the period of 8 years, 31[a] patients with

[a] Three leukemic individuals were not included in the study due to the profession in 2, one being a furniture worker and the second working in a printing shop. The third individual was not included because his working place was outside of Istanbul.

Material and Methods

According to the official records there are 28 500 workers involved in the shoe slipper and handbag industry in Istanbul [3] The working conditions, including the mode of benzene exposure are described elsewhere [3, 6, 7] In Istanbul during the period from 1967 to 1974, 33 leukemic individuals chronically exposed to benzene[1] were found With the exception of 2 (1 furniture worker, 1 working in a printing shop), all were shoe-workers Twenty six of these patients were described elsewhere [3-5] In 1974 8 new leukemic patients with chronic exposure to benzene were studied in the Internal Clinic of Istanbul Medical School The concentrations of benzene in their working environment measured by a Drager-Spürgerät Multi Gas Detector Mod 21 23, Lubek, were recorded to reach 210 or, rarely, 640 ppm when benzene containing adhesives were used

Results

Some clinical and hematological findings and the duration of exposure are given in tables I and II

Types of leukemia The comparison of the distribution of the types of leukemia found in these 34 individuals chronically exposed to benzene and those of 50 non exposed leukemic patients is given in table III Similarly, the ages of these non-exposed leukemic individuals who have been studied in our clinic during the last $1\frac{1}{2}$ years varied between 16 and 60 years with a mean of 37 7 years According to our results, acute myeloblastic leukemia seems to be the most frequent variety of this malignancy following exposure to benzene Then comes preleukemia, erythroleukemia and acute lymphoblastic leukemia The criteria for the diagnosis of preleukemia were similar to those of WINTROBE *et al* [20] (1) the presence of refractory anemia, mostly hypochromic or pancytopenia, (2) the percentages of myeloblasts in the bone marrow within the normal range or slightly increased, and (3) the presence of a few blast cells, mostly myeloblasts, in the peripheral blood film

Pancytopenic period A preceding pancytopenic period was present in 23 5% of all cases It was noted in eight patients, three with acute myeloblastic leukemia, three with preleukemia, one with acute monocytic leukemia and one with acute erythroleukemia Preceding pancytopenic period was particularly present in 50% of preleukemic patients The interval between the onset of leukemia and that of pancytopenic period varied from 6 months to 6 years It was the shortest in case 5 and the longest in

[1] One of the 34 leukemic patients was exposed to benzene outside of Istanbul

Table IV Annual number of leukemic shoe-workers in Istanbul between 1967 and 1974

Years	Number of leukemic shoe-workers	Years	Number of leukemic shoe-workers
1967	1	1971	6
1968	1	1972	5
1969	3	1973	7
1970	4	1974	4

acute leukemia, one lymphoblastic and the other myeloblastic. This family was described elsewhere [5] The third patient, case 8 of the series presented here had acute erythroleukemia His father, a 65-year-old shoe-worker with a long history of benzene exposure died nearly 1 year ago in a hospital in Istanbul with the diagnosis of myelosclerosis But re-evaluation of the case report showed that this patient possibly had an acute leukemia of unidentified type The disease showed an acute course with severe gastrointestinal bleedings and profound anemia and there was no hepatosplenomegaly Four sternal punctures yielded no material

Some contributary factors for the development of leukemia with a preceding pancytopenic period In 6 of 8 new patients with acute leukemia, corticosteroids were used during the course of pancytopenic period varying from some months to several years In these pancytopenic patients, acute leukemia or preleukemia developed with an interval of 6 months to 6 years In addition to that, re-exposure to benzene was also noted in 4 of these 6 patients

Discussion

Since the first case report of leukemia due to chronic exposure to benzene appeared [17], numerous leukemic patients with chronic exposure to this chemical were described As an example, BROWNING [9] collected 61 cases of leukemia with chronic benzene poisoning from the literature till 1965 More than that appeared in the literature since that time The establishment of a marked and statistically significant increase in the incidence of leukemia among shoe workers chronically exposed to benzene by this study and previous works [3] provided final evidence of the leukemogenic effect of benzene in man

Types of leukemia. Thirty-three of 34 leukemic individuals of this series had various types of acute leukemia. Interestingly, there was only 1 case of chronic leukemia, myeloid in type. According to this study, acute myeloblastic leukemia seems to be the most frequently encountered type of leukemia following chronic exposure to benzene. This finding is in accordance with those of VIGLIANI and SAITA [19]. In this study a striking increase in the number of the patients with preleukemia (6 cases, 17.6%) and acute erythroleukemia (6 patients, 17.6%) was noted. In this respect, with small differences, the frequency of various morphologic types of acute leukemia in our series was similar to that of GOGUEL *et al.* [13], obtained in 23 workers with acute leukemia due to chronic exposure to benzene. As noted above, in our series only 1 case of chronic myeloid leukemia was present. In contrast to our results, there are several reports describing the occurrence of chronic myeloid or even chronic lymphoid leukemia in the patients with chronic benzene poisoning. BROWNING [9] collected 21 cases of chronic myeloid and 6 cases of chronic lymphoid leukemia among 61 leukemic individuals with chronic benzene exposure. Furthermore, in 1967 GOGUEL *et al.* [13] noted that there were 16 cases with chronic myeloid and 8 with chronic lymphoid leukemia in their series of leukemic patients with exposure to this chemical. Therefore, it is difficult to explain the significant difference existing between the distribution of the types of leukemia due to chronic benzene poisoning established in our series and in the group arranged by BROWNING [9] and that of GOGUEL *et al.* [13]. On the other hand, if we compare the types of leukemia occurring in 34 exposed individuals and 50 non-exposed persons a striking difference can be seen between these two groups of leukemic patients. This result is in contrast to that observed among heavily irradiated survivors in Hiroshima and Nagasaki [8, 14–16]. The occurrence of chronic myeloid leukemia was frequent among these groups of survivors. Furthermore, a small but significant difference was found concerning the incidence of chronic myeloid leukemia in atomic bomb survivors in Hiroshima and Nagasaki, 33 and 18%, respectively [15]. This difference in the incidence rate of chronic myeloid leukemia was found to be related to the dose and the nature of the radiation [15]. On the other hand, the almost absence of chronic lymphoid leukemia among these groups of survivors of atomic bomb was explained by the infrequent occurrence of chronic lymphoid leukemia in Japan [16]. But in Turkish people, the distribution of the types of leukemia is almost similar to that observed in Western countries. Therefore, the absence of chronic lymphoid leukemia and the rarity of

chronic myeloid leukemia in our series is significant. On the other hand, our results are somewhat similar to those of COURT BROWN and DOLL [11, 12] obtained in 50 patients receiving partial body irradiation for treatment of ankylosing spondylitis. In this study, 38 out of 50 cases had acute leukemia and only 8 patients with chronic forms, 7 myeloid and 1 lymphoid, have been found. According to this study, the data in the remaining cases were insufficient to determine the clinical type of this disease. Furthermore, there are numerous cases of leukemia following radio iodine treatment [1, 2, 10, 18]. With the exception of 2 examples of chronic myeloid leukemia [2, 18] all patients had acute leukemia.

The foregoing data show that the distribution of the types of leukemia following exposure to benzene varied significantly between studies [3, 9, 13, 19]. A certain relationship between the duration of exposure and the types of leukemia was noted in the present study (table II).

Finally, the role of genetic predisposition was evident in 3 of our leukemic patients in 2 families. In the first family described previously elsewhere [5], uncle and paternal nephew had acute leukemia: one lymphoblastic, the other myeloblastic. In the second family, the father had acute leukemia of unidentified type and his son had acute erythroleukemia. The occurrence of leukemia in these two families was more than a mere coincidence. Therefore, in our opinion, some genetic factors triggered by chronic exposure to benzene played a role in these two families with acute leukemia.

References

1 ABBAT, J. D.; FARRAN, E. A., and GREENE, R.: Acute myeloid leukaemia after radioactive iodine (I^{131}) therapy. Lancet *i*: 782 (1956).

2 AKSOY, M.: Chronic myeloid leukemia after radioiodine therapy for hyperthyroidism. Med. Bull. Istanbul *6*: 185 (1973).

3 AKSOY, M.; ERDEM, Ş., and DINÇOL, G.: Leukemia in shoe workers exposed chronically to benzene. Blood *44*: 837 (1974).

4 AKSOY, M.; DINÇOL, K.; ERDEM, Ş., and DINÇOL, G.: Acute leukemia due to chronic exposure to benzene. Am. J. Med. *52*: 160 (1972).

5 AKSOY, M.; ERDEM, Ş.; ERDOĞAN, G., and DINÇOL, G.: Acute leukaemia in two generations following chronic exposure to benzene. Hum. Hered. *24*: 70 (1974).

6 AKSOY, M.; DINÇOL, K.; AKGÜN, T.; ERDEM, Ş., and DINÇOL, G.: Haematological effects of chronic benzene poisoning in 217 workers. Br. J. ind. Med. *28*: 296 (1971).

7 AKSOY, M.; DINÇOL, K.; ERDEM, Ş.; AKGÜN, T., and DINÇOL, G.: Details of blood changes in 32 patients with pancytopenia associated with long term exposure to benzene. Br. J. ind. Med. *29*: 56 (1972).

8 BIZZOZERO, G J jr, JOHNSON, K G., and CIOCCO, A Radiation related leukemia in Hiroshima and Nagasaki 1946–1964 I Distribution, incidence and appearance in time New Engl J Med *274* 1095 (1966)
9 BROWNING, E Toxicity and metabolism of industrial solvents, p 41 (Elsevier, Amsterdam 1965)
10 BURNS T W, VICKERS R., and LOWNEY, J F Acute leukemia after radioactive iodine (I^{131}) therapy for hyperthyroidism Archs intern Med *106* 97 (1960)
11 COURT BROWN, W M and DOLL, R Leukaemia and aplastic anaemia in patients irradiated for ankylosing spondylitis Spec Rep Ser med Res Coun, No 295 (1957)
12 COURT BROWN, W M and DOLL, R Adult leukaemia incidence in mortality in relation to aetiology Br med J *I* 1063 (1959)
13 GOGUEL, A, CAVIGNEAUX, A et BERNARD, J Les leucémies benzéniques Bull Inst nat Santé *22* 421 (1967)
14 HEYSSEL, E., BRILL, A R, WOODBURY, L. A, NISHIMURA, E T, GHOSE, T, HOSHINO, T, and YAMASAKI, M Leukemia in Hiroshima atomic bomb survivors Blood *15* 313 (1960)
15 HOSHINO, T Etiologic role of ionizing radiation on the development of leukemia clinical and statistical studies on leukemia in patients treated with radioiodine and in atomic bomb survivors Acta haemat jap *31* 825 (1968)
16 LANGE, R D, MOLONY, W C, and YAMAWAKI T Leukemia in atomic bomb survivors I General observations. Blood *9* 574 (1954)
17 LE NOIRE et CLAUDE Sur un cas de purpura attribué a l intoxication par le benzène Bull Mém Soc méd Hôp Paris *14* 1251 (1897), cité par BROWNING [9]
18 LIVERGANT, Y E. and MAKHANIA, M S A combination of chronic myeloleukemia with thyreotoxicosis subjected to radioiodine therapy Med Radiol, Moscow *10* 26 (1964)
19 VIGLIANI, E C and SAITA G Benzene and leukemia New Engl J Med *271* 872 (1964)
20 WINTROBE, M M, LEE, G R, BOGGS, D R, BITHELL, T C, ATHENS, J W, and FOERSTER, J Clinical hematology, p 1477 (Lea & Febiger, Philadelphia 1974)

Prof M AKSOY Section of Hematology, Department of Internal Medicine, Istanbul Medical School Çapa Istanbul (Turkey)

Acta haemat. 55 73–80 (1976)

Different Composition and Mitotic Activity of the Haemopoietic Tissue in Bone Marrow, Spleen and Liver in Chronic Myeloid Leukaemia

U. SJÖGREN and L. BRANDT

Department of Internal Medicine, University Hospital, Lund

Key Words Bone marrow Extramedullary haemopoiesis Liver Myeloid leukaemia Spleen.

Abstract Aspirates from bone marrow, spleen and liver were analyzed in 15 untreated patients with chronic myeloid leukaemia (CML). The proportion of erythroblasts was higher in the spleen and liver than in the bone marrow. An increased frequency of basophilic leucocytes was recorded in the extramedullary sites compared to the bone marrow. In the liver aspirates a considerable admixture of lymphocytes was found. The mitotic indices of the granulopoietic and erythropoietic precursor cells were lower in the spleen and liver than in the bone marrow. The results indicate that the composition and proliferative activity of the haemopoietic tissue differ in bone marrow and extramedullary sites in CML. The discrepancies may be due to differences in the microenvironment for the haemopoietic cells or to a selective accumulation of cells with a low mitotic activity in extramedullary sites. Since the final blastic transformation of CML may originate outside the bone marrow, further studies of the extramedullary haemopoietic tissue may be worthwhile.

The chronic phase of chronic myeloid leukaemia (CML) is characterized by a successive increase of intra- and extramedullary haemopoietic cells. Although a reduction of the blood forming tissue may be temporarily achieved due to treatment, the final blastic transformation usually develops in patients with considerable hepatomegaly and splenomegaly and whose bone marrow is overcrowded with cells.

Practically no information is available on the composition and function of the haemopoietic tissue in the liver in CML but there are several indications that the blood forming tissue of the spleen and the bone marrow may differ in various respects. Thus, in the chronic phase of the disease, discrepancies in the composition and kinetic properties of the granulopoietic tissue have been demonstrated [4, 5, 7, 21] and differences between intramedullary and splenic erythropoietic tissue have also been found [24]. The

significance of these differences is far from clear but since it has been demonstrated that morphologic or cytogenetic signs of the terminal blastic crisis may be detected in the spleen [19–22], in lymph nodes [10, 12, 15, 17, 28] as well as in other extramedullary sites [14, 16] prior to such changes in the bone marrow, it may be worth-while to study the extramedullary blood forming tissue more extensively in CML

The present investigation was undertaken in order to compare the composition of the haemopoietic tissue in liver and spleen to that of the bone marrow in untreated patients with CML In addition the mitotic activity of the granulocytic and erythroid precursors was compared in the three organs

Material and Methods

Patients 15 untreated patients with a recent diagnosis of CML were examined The WBC counts were 76,000–311 000 No patient was in blastic crisis

Morphological investigations Bone marrow was obtained through sternal puncture and material from the liver and spleen through fine-needle aspiration biopsy as described by Söderström [26] Aspirates from the three organs were obtained on the same occasion and smears were stained with May Grünwald Giemsa Through examination of 1,000–3,000 (mean 1,100) nucleated cells the percentages of granulocytic cells at different stages of maturation, erythroblasts, basophilic leucocytes and lymphocytes were determined Neither cells belonging to the liver parenchyma nor splenic lymphocytes or pulp cells were included in these calculations A mitotic index of the granulopoietic precursor cells (myeloblasts + promyelocytes + myelocytes) was determined in each smear through examination of 1,600–7,500 (mean 3 800) such cells and a mitotic index of the erythroid precursor cells through examination of 500–1,800 (mean 950) erythroblasts At the same time the erythroblasts were morphologically classified Proerythroblasts and basophilic erythroblasts were then pooled into one group designated basophilic erythroblasts

Statistics The Wilcoxon matched pairs signed ranks test was used to assert significance of the results

Results

Composition of intra- and extramedullary haemopoietic tissue The median proportion of erythroid precursors in the bone marrow was 3 6% In the spleen the corresponding figure was 7 5% which is significantly higher ($p<0\ 02$) A higher median percentage of erythroblasts, 6 7%, was also recorded in the liver although the difference between the bone marrow and the liver aspirates was not fully significant ($p<0\ 06$) (fig 1)

In the bone marrow on an average 3 0% of the cells were basophilic leucocytes compared to 4 7% in the spleen and 4 8% in the liver The

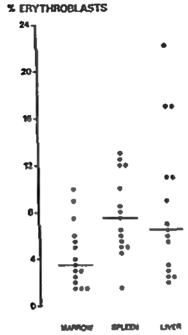


Fig 1 Percentage of erythroblasts within the haemopoietic tissue of bone marrow spleen and liver in 15 patients with CML. Median values are indicated

increase of basophilic cells was significant ($p<0.01$) in both spleen and liver when compared to the bone marrow (fig 2)

The percentage of lymphocytes was 0 3–3 5% (median 1 3%) in the bone marrow and 3 6–17 1% (median 6 4%) in the liver The difference is significant ($p<0.01$)

Mitotic activity The median mitotic index of the granulopoietic precursor cells was 1 07% in the bone marrow, 0 70% in the spleen and 0 64% in the liver (fig 3) The mitotic indices in both extramedullary sites were significantly lower than in the bone marrow ($p<0.01$) There were no indications that the discrepancies in mitotic activity were due to the presence of different amounts of mature neutrophils known to produce substances that interfere with the proliferation of granulopoietic precursors [23] Thus the percentage of mature neutrophils was on an average 35 0% in the bone marrow, 38 3% in the liver and 29 5% in the spleen

The mitotic indices of the erythroblasts were likewise significantly lower ($p<0.01$) in the spleen and liver than in the bone marrow the median values

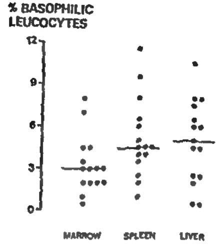


Fig 2 Percentage of basophilic leucocytes in bone marrow, spleen and liver

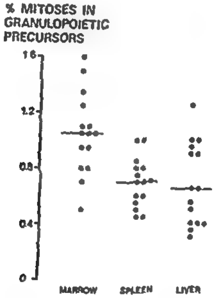


Fig 3 Mitotic indices of granulopoietic precursor cells in bone marrow, spleen and liver

being 2 5, 2 6 and 3 5%, respectively (fig 4) The higher indices in the bone marrow were not caused by an unduly large proportion of early erythroid cells known to have higher mitotic indices than the more mature erythroblasts Thus the average proportion of basophilic erythroblasts within the erythropoietic pool in the marrow was 37 8% compared to 38 4% in the spleen and 40 0% in the liver

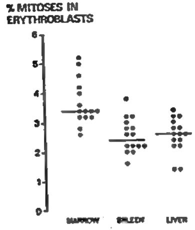


Fig 4 Mitotic indices of the erythroblasts in bone marrow, spleen and liver

Discussion

The present data indicate that several characteristics are common to extramedullary haemopoietic tissue whether situated in the spleen or the liver in CML. Furthermore, in several respects the extramedullary tissue was found to differ from the bone marrow. It has previously been demonstrated that in CML the proportion of erythroblasts within the haemopoietic tissue is larger in the spleen than in the bone marrow and that the increasing splenomegaly during the course of the disease therefore means a considerable contribution of erythroid precursors [24]. The present data indicate that the extramedullary tissue in the liver is also relatively rich in erythroid cells. It is known that haemodilution and sequestration of red cells in the enlarged spleen contribute to the development of anaemia in CML [2, 3, 9]. The low mitotic activity of the spleen and liver erythroblasts may help to explain why the large amount of extramedullary erythropoietic tissue in advanced CML obviously fails to compensate factors that promote the development of anaemia.

A low mitotic activity was also found in the granulopoietic precursors in spleen and liver. An accumulation in the bone marrow of early granulopoietic precursor cells with a low mitotic activity at diagnosis of CML has been found to indicate a serious prognosis with a short life span, i.e. the patient may be near the final blastic crisis [25]. Furthermore, in Ph¹-chromosome-negative CML, known to generally run a more rapid malignant

course than Ph¹-chromosome-positive CML [11], the mitotic indices of the granulopoietic precursor cells have been found to be extraordinarily low [6]. It may therefore be that a low mitotic activity of granulopoietic precursors is a sign of 'leukaemicness' in CML and if so the prevalence of precursors with a low mitotic activity found in the spleen and liver may indicate that these organs lodge cells with even more malignant properties than the bone marrow. In this context it is of interest to note that the mitotic indices of the granulopoietic precursors of the spleen and liver in the present cases were often as low as those generally found in bone marrow blast cells of a group of patients with acute myeloid leukaemia recently studied by us [8]. Furthermore, in two CML patients karyotypic changes characteristic of the blastic transformation have been found in the spleen before they could be detected in the bone marrow, which may likewise indicate an increased tendency to malignant behaviour of extramedullary haemopoietic tissue in CML [19, 20]

The cause of the characteristic increase of basophilic leucocytes in chronic myeloproliferative disorders is unknown. In the present CML patients the basophilic leucocytes were significantly more common within the haemopoietic tissue of the spleen and liver than in the bone marrow. It is known that basophilic leucocytes will increase in tissues where cell- [illegible] that the [illegible] cytes [27]. It is therefore possible that the overrepresentation of basophilic leucocytes in the liver and spleen in CML reflects a cell-mediated immunological response against leukaemic cells occurring in these organs. If haemopoietic tissue in spleen and liver is more prone to malignant transformation than the bone marrow, it is conceivable that immune response would be more pronounced in the extramedullary sites. KAUR *et al.* [13] have demonstrated an increase of T lymphocytes in the spleen in CML which is in agreement with such an interpretation, since their findings suggest that cell-mediated immune reactions against leukaemic cells may take place in that organ. The admixture of lymphocytes was significantly higher in the liver specimens than in the bone marrow of our patients. It is known that liver biopsy specimens from normals contain considerable numbers of lymphocytes [18, 26] and it is therefore uncertain whether the relatively high proportions of these cells found in the liver represented normally occurring lymphocytes or were caused by an accumulation of lymphoid cells in the liver. Nevertheless the results indicate the presence of a considerable amount of lymphocytes in the liver in CML, and it cannot be ruled out that these cells may exert immune reactions similar to those in the spleen.

Even if the present results together with results of previous cytogenetic investigations might suggest that extramedullary tissue in CML is more prone to malignant transformation than bone marrow tissue, the reason for this discrepancy remains obscure It is, however, apparent that the environment for the haemopoietic tissue is different in bone marrow and extramedullary sites It is possible that at initiation of CML the haemopoietic cells are programmed for a development ending up in the blastic transformation and that an unfavourable microenvironment for the blood-forming cells in extramedullary sites may promote this development Another possibility is that during the chronic phase of CML circulating cells with potential malignant properties are more or less selectively trapped in extramedullary organs and there may give rise to immune reactions and undergo the final blastic transformation

References

1 Aspegren, N, Fregert, S, and Rorsman, H Basophil leukocytes in allergic eczematous contact dermatitis. Int. Archs Allergy appl. Immun *23* 150–156 (1963)

2 Blendis, L. M, Ramboer, C., and Williams, R. Studies on the haemodilution anaemia of splenomegaly Eur J clin Invest. *1* 54–64 (1970)

3 Bowdler, A J Blood volume studies in patients with splenomegaly Transfusion *10* 171–181 (1970)

4 Brandt, L. Difference in the proliferative activity of myelocytes from bone marrow, spleen and peripheral blood in chronic myeloid leukaemia Scand J Haematol *6* 105–112 (1969).

5 Brandt, L. Difference in uptake of tritiated thymidine by myelocytes from bone marrow and spleen in chronic myeloid leukaemia Scand. J Haematol. *11* 23–26 (1973)

6 Brandt, L., Mitelman, F, Nilsson, P G, and Sjögren, U Low mitotic activity of granulopoietic precursor cells in Ph^1-chromosome-negative chronic myeloid leukaemia Lancet II 719–720 (1974).

7 Brandt, L. and Schnell, C. R. Granulopoiesis in bone marrow and spleen in chronic myeloid leukaemia Scand. J Haematol. *6* 65–63 (1969)

8 Brandt L. Mitelman, F, and Sjögren, U Relation between chromosomes and mitotic activity in acute myeloid leukemia. Hereditas 79 305–306 (1975)

9 Christensen, B E. Erythrocyte pooling and sequestration in enlarged spleens. Estimations of splenic erythrocyte and plasma volume in splenomegalic patients. Scand. J Haematol *10* 106–119 (1973)

10 Duvall, C. P Carbone, P P, Bell, W R., Whang, J, Tjio, J H., and Perry, S Chronic myelocytic leukemia with two Philadelphia-chromosomes and prominent peripheral lymphadenopathy Blood 29 652–666 (1967).

11 Lednicky, E. Z. Sokal, J E., Crosswhite, L., and Sandberg, A. A. Philadelphia-

chromosome-positive and negative chronic myelocytic leukemia Ann intern Med *72* *175–182 (1970)*

12 GARFINKEL, L S and BENNETT, D E Extramedullary myeloblastic transformation in chronic myelocytic leukemia simulating a coexistent malignant lymphoma. Am. J clin Path *51* 638–645 (1969)

13 KAUR, J, CATOVSKY, D, SPIERS A S D, and GALTON, D A G Increase of T lymphocytes in the spleen in chronic granulocytic leukaemia Lancet *I* 834–836 (1974)

14 KILLMANN, S A Chronic myelogenous leukemia Preleukemia or leukemia?, in TURA and BACCARANI Chronic myeloid leukaemia pp 45–53 (Edizioni di Haematologica, Pavia 1972)

15 KNOSPE, W H, KLATT, R W, BERGIN, J W, and JACOBSON, C B Cytogenetic changes in chronic granulocytic leukaemia during blast crisis Two Ph¹-chromosomes and hyperdiploidy *Am J med Sci 254 816–823 (1967)*

16 KWAAN, H C, PIERRE, R V, and LONG, D L Meningeal involvement as first manifestation of acute myeloblastic transformation in chronic granulocytic leukemia Blood *33* 348–352 (1969)

17 LIBRE, E P and MCFARLAND, W *Chronic myelogenous leukemia* Possible association with reticulum cell sarcoma Archs intern Med *119* 626–630 (1967)

18 LUNDQUIST, A. and ÅKERMAN, M Fine-needle aspiration biopsy in acute hepatitis and liver cirrhosis Ann clin Res *2* 197–203 (1970)

19 MITELMAN, F, BRANDT, L., and NILSSON, P G Cytogenetic evidence for splenic origin of blastic transformation in chronic myeloid leukaemia Scand J Haematol *13* 87–92 (1974)

20 MITELMAN, F, NILSSON, P G, and BRANDT, L. Abnormal clones resembling those seen in blast crisis arising in the spleen in chronic myelocytic leukemia J natn Cancer Inst (in press, 1975)

21 MOESCHLIN, S Spleen puncture, pp 130–140 (Heinemann, London 1951)

22 ROHR, R *Das menschliche Knochenmark*, p *113* (Thieme, Stuttgart 1949)

23 RYTÖMAA, T Annotation Role of chalone in granulopoiesis Br J Haemat *24* 141–146 (1973)

24 SJÖGREN, U and BRANDT, L. Differences in morphology and mitotic activity between intra and extra medullary erythropoietic tissue in chronic myeloid leukaemia Scand J Haematol *13* 116–120 (1974)

25 SJÖGREN, U, BRANDT, L, and MITELMAN, F Relation between life expectancy and composition of the bone marrow at diagnosis of chronic myeloid leukaemia. *Scand J* Haematol *12* 369–373 (1974)

26 SÖDERSTRÖM, N Fine-needle aspiration biopsy Used as a direct adjunct in clinical diagnostic work (Grune & Stratton, New York 1966)

27 WOLF JÜRGENSEN, P and SCHWARTZ, M Normal lymphocyte transfer in man Basophil leucocytes in delayed skin reaction Lancet *II* 388–390 (1964)

28 WOODSON D L, BENNETT, D E, and SEARS, D A Extramedullary myeloblastic transformation of chronic myelocytic leukemia. Lymph node infiltration *one year before marrow blastic change* Archs intern Med *134* 523–526 (1974)

Dr U SJÖGREN, Department of Internal Medicine, University Hospital, *S-221 85 Lund* (Sweden)

Acta haemat 55 81-88 (1976)

Leukozytenlipide bei reifzelligen Leukämien

RENATE BLEIBER, D KUNZE, G REICHMANN und E HOFER

Hämatologische Abteilung (Leiter Prof H STOBBE) der I Medizinischen Universitätsklinik (Direktor Prof F H SCHULZ) und Abteilung für Klinische Biochemie (Leiter Prof E. EGGER) des Bereichs Medizin, Humboldt Universität, Berlin

Key Words Leukemic leukocytes Leukocyte lipids Lipids in leukemia

Abstract The total lipid content and the lipid pattern of normal and leukemic leukocytes showed no significant difference except the fact, that lymphocytes of chronic lymphatic leukemia have a lower content of triglycerides and sphingomyeline Lecithin of leukemic lymphocytes contained more palmitinic acid and oleic acid and a lower level of stearic acid than the same fraction in normal lymphocytes. The lipid pattern of normal and leukemic granulocytes was identical

Da die Lipide einen wesentlichen Bestandteil der Zellmembran darstellen, wurden lipidanalytische Untersuchungen auch an Tumorzellen wiederholt vorgenommen, zumal bestimmte Tumorzelleigenschaften, wie die Antigenität, Kontaktinhibition, Adhäsivität, Permeabilität, Oberflächenladung u a , gegenüber normalen Zellen verändert sind und damit auf Abweichungen im Zellmembranaufbau hinweisen [1, 3, 6, 12, 17–20] Art und Zustandekommen dieser Veränderungen, ihr Zusammenhang mit der neoplastischen Umwandlung und die funktionellen Auswirkungen auf die Zelle sind im einzelnen meist unklar

Wegen der Möglichkeit, weitgehend reine Zellanreicherungen zu gewinnen, sind Blutzellen für vergleichende Untersuchungen am Menschen besonders geeignet

Bereits vor Jahrzehnten erfolgten Gegenüberstellungen des Lipidgehaltes normaler und leukämischer Leukozyten Erst seit etwa 15 Jahren sind jedoch aus methodischen Gründen reproduzierbare Ergebnisse

möglich. In diesem Zeitraum wurden vor allem von KIM *et al.* [8], SCHWANDT *et al.* [15, 16] und GOTTFRIED [4, 5] vergleichende Untersuchungen leukämischer Leukozyten durchgeführt. Nur GOTTFRIED zog zum Vergleich gereinigte normale Leukozytenpopulationen heran, während die anderen Untersucher Gesamtleukozytenanreicherungen verwandten. Unter Bezug auf die Zellzahl fand GOTTFRIED bei allen neoplastischen Leukozyten, aber auch bei langfristig kultivierten Normalzellen gleichartige Veränderungen des Zellipidgehaltes, nämlich Verminderung des Gesamtlipids, Verminderung des Cholesterins, relative Vermehrung der Phospholipide. Innerhalb der Phospholipide waren Sphingomyelin vermindert und Phosphatidylcholin vermehrt. Diese Veränderungen wurden von ihm als unspezifisches Zeichen der Unreife gedeutet und nicht mit der neoplastischen Umwandlung in Zusammenhang gebracht.

Die vorliegende Arbeit untersucht Lipidgehalt, Lipidverteilung und Fettsäuremuster von Leukozyten bei reifzelligen Leukosen. Unter Benutzung der Eiweissmenge als Bezugsgrösse konnten bei der Gegenüberstellung mit normalen gereinigten Leukozytenpopulationen sichere Abweichungen nur bei der lymphatischen Leukose nachgewiesen werden. Die Differenzen zwischen normalen und leukämischen Granulozyten waren meist nur geringfügig und statistisch nicht zu sichern.

Material und Methodik

Ausgangsmaterial waren jeweils 40 ml frisches Zitratblut. Die Isolierung von Lymphozyten und Granulozyten erfolgte nach der von OTTO und SCHMID [13, 14] beschriebenen Methodik. Anstelle von Uromiro® wurde Visotrast® auf das erforderliche spezifische Gewicht eingestellt.

Für die Kontrollgruppe wurde Blut Blutgesunder aufbereitet, wobei Lymphozyten- bzw. Granulozytenanreicherungen von jeweils 5–10 Patienten zusammengenommen und als eine Einzelprobe verarbeitet wurden. Die Lipidanalyse der Leukosezellen erfolgte aus Einzelblutproben von Patienten, die keine zytostatische Behandlung erhielten und eine Leukozytenzahl von mindestens [illegible]000/µl aufwiesen. Die Lipidextraktion erfolgte nach FOLCH. Die Homogenisierung der Zellen wurde in Chloroform-Methanol (2:1) mit dem Ultraturrax vorgenommen. Nach Filtration und Einengung wurden die Lipide dünnschichtchromatographisch getrennt, Neutralfette im System Petroläther/Äther/Eisessig, Phosphatide im System Chloroform/Methanol/Wasser.

Nach Abtragen der mit Jod bzw. Rhodamin gefärbten Banden wurden die Fraktionen einzeln bestimmt. Cholesterin und Cholesterinester nach BEUKERS *et al.* [2], Triglyzeride mit der Sulphophosphovanillinreaktion nach ZÖLLNER und EBERHAGEN

HAGEN [21], die Phosphatide nach Veraschung mit Perchlorsäure als Orthophosphat nach MARTIN und DOTY [10]. Alle Methoden wurden auf die Anwendung auf Kieselgel adaptiert, eine ausführliche Darstellung erfolgte bereits [7]. Als Bezugsgrösse diente der Gehalt an Eiweiss, die Eiweissbestimmung wurde nach LOWRY *et al.* [9] vorgenommen.

An normalen und leukämischen Lymphozyten wurde eine Fettsäureanalytik der Phospholipide durchgeführt. Hier wurde sämtlichen bei der Phospholipidextraktion und -trennung verwendeten Lösungsmitteln das Antioxydans BHT zugesetzt. Die dünnschichtchromatographische Phospholipidtrennung erfolgte in der Modifikation einer Zweischritttrennung. Als erstes Laufmittel wurde Chloroform/Methanol/Wasser (150:50:8), als zweites Laufmittel Petroläther/Äther (180:20) verwandt.

Nach Anfärben der Lipidbanden mit 2,7-Difluoreszein wurde die Kieselgelzone abgetragen und unter N_2 ampulliert. Die Umesterung der Fettsäuren zu den Methylestern erfolgte mit methanolischem BF_3 entsprechend den Angaben von MORRISON und SMITH [11].

Die gaschromatographische Trennung wurde mit einem Gaschromatographen der Firma Varian Aerograph, Modell 2100, ausgerüstet mit einem Flammenionisationsdetektor und einem elektronischen Integrator (Modell 475) der gleichen Firma durchgeführt.

Die Säulenfüllung erfolgte mit 10% EGSS-X auf Gaschrom-Q-100-120 mesh. Für die Identifizierung der einzelnen Fettsäuren wurde isotherm bei 180 °C gearbeitet, für die quantitative Bestimmung wurde die Temperatur programmiert von 150–190 °C mit einer Heizrate von 1 °C/min. Die quantitative Auswertung wurde mit den NIH-Testsubstanzen kontrolliert und gewährleistet. Die statistische Berechnung erfolgte nach Prüfung der Normalverteilung mit dem t-Test.

Ergebnisse

Der mittlere Lipidgehalt pro Milligramm Eiweiss lag nach Addition der Einzelfraktionen bei normalen Lymphozyten bei 421,6 nmol, bei leukämischen Lymphozyten bei 440,7 nmol. Ein statistisch sicherer Unterschied bestand zwischen beiden Werten nicht. Normale Granulozyten enthielten 693,9 ± 121,7 nmol Lipid/mg Eiweiss, leukämische Granulozyten 482 ± 112,2 nmol/mg Eiweiss. Der Unterschied zwischen beiden Leukozytengruppen war nicht signifikant (t = 1,4).

Der Phospholipidanteil am Gesamtlipid bewegte sich zwischen 68 und 77%. Es ergaben sich keine signifikanten Abweichungen zwischen normalen und leukämischen Zellen.

An Neutralfetten enthielten normale Leukozyten prozentual mehr Triglyzeride als leukämische. Dieser Unterschied war jedoch nur bei den Lymphozyten signifikant (Tabelle I).

[illegible]

[illegible] und Granulozyten

	[illegible]		[illegible]		[illegible]	
		s	$\bar{x}$	s	$\bar{x}$	s
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	36,3	[illegible]

[illegible] zu den Gesamt[illegible] in [illegible] und Granulozyten

[illegible]	PC		PÄ		PS – PG	
	$\bar{x}$			s	$\bar{x}$	s
[illegible]					[illegible]	[illegible]
	[illegible]					
	9,9					
	5,2					

Sphingomy[illegible]
= Phospha[illegible]

[illegible]

Fettsäuren	Normal		Leukämisch	
	$\bar{x}$	s	$\bar{x}$	s
Phosphatidylcholin				
16 0	14,0	7,2	41,9	1,87[1]
18 0	53,8	1,14	20,1	2,63[1]
18 1	13,9	1,9	20,6	1,48[1]
18 2	3,5	0	4,27	2,94
20 0	1,78	0,78	0,63	0,11
20 4	4,7	0	3,65	2,6
Phosphatidyläthanolamin				
16 0	7,16	1,5	12,2	
18 0	42,7	20,6	45,0	
18 1	9,49	4 67	17,3	
18 2	4,36	2,0	1,85[2]	
20 0	1,36	1,97	0,52	
20 4	3,09	1,58	3,59	
Sphingomyelin				
16 0	14,1	2,2	16,4	
18 0	49,7	0	48,5	
18 1	4,96	1,4	9,7[2]	
18 2	10,1	2,6	0,6	
20 0	1,0	0,4	7,8	
20 4	1,2	0,4	0,5	

[1] $p<0{,}05$
[2] Einzelbestimmungen

Normale und leukämische Lymphozyten wiesen eine unterschiedliche Phospholipidverteilung auf. Leukämische Lymphozyten enthielten signifikant weniger Sphingomyelin als normale. Eine entsprechende Lezithinvermehrung war statistisch nicht zu sichern. Gleichartige Veränderungen konnten an den Granulozyten nicht nachgewiesen werden (Tabelle II)

Wegen der Unterschiede der Phospholipidverteilung wurde bei normalen und leukämischen Lymphozyten eine Fettsäureanalyse durchgeführt Dabei enthielt das Lezithin leukämischer Lymphozyten

signifikant mehr Palmitin- und Ölsäure, die gleiche Fraktion normaler Lymphozyten mehr Stearinsäure (Tabelle III)

Diskussion

Aus unseren Befunden ist die Schlussfolgerung zu ziehen, dass in Relation zum Eiweissgehalt keine signifikanten Veränderungen im Gesamtlipidgehalt bei Vergleich normaler und leukämischer Zellen nachweisbar sind und die absoluten mittleren Lipidmengen, bezogen pro Milligramm Eiweiss bei normalen und leukämischen Lymphozyten etwa gleich hoch, bei leukämischen Granulozyten leicht, jedoch nicht signifikant vermindert sind Normale Granulozyten enthalten am meisten, normale Lymphozyten am wenigsten Lipid Vergleiche zwischen leukämischen Lymphozyten und normalen Gesamtleukozytenanreicherungen, die vorwiegend Granulozyten enthalten, sind damit nicht möglich

Bis auf die relative Sphingomyelinverminderung die auch wir bei leukämischen Lymphozyten nachweisen konnten – bei leukämischen Granulozyten bestanden ähnliche Veränderungen nicht – weichen unsere Befunde von denen von GOTTFRIED [4, 5] ab Dies möchten wir auf unterschiedliche Bezugsgrössen zurückführen Ein Zugrundelegen der Zellzahl kann z B Zellgrossen- und Strukturveränderungen nicht berücksichtigen, ermöglicht dagegen eine Aussage über den Lipidgehalt der Einzelzelle

Der Bezug auf Eiweiss vergleicht mit einem biochemischen Parameter der mit gewissen Einschränkungen als Mass der Stoffwechselaktivität gesehen werden kann

Das Fettsäuremuster der Phospholipide bestimmt weitgehend deren physiologische Eigenschaften und scheint für wichtige Membraneigenschaften (Aktivität membranständiger Enzyme, Permeabilität u a) von ausschlaggebender Bedeutung zu sein Die zur Verfügung stehenden Materialmengen erlaubten lediglich Einzelbestimmungen der Fettsäuren von Phosphatidyläthanolamin und Sphingomyelin leukämischer Lymphozyten Die ersichtlichen Unterschiede im Phosphatidyläthanolamin bei 16 0, 18 1 und 18 2, in Sphingomyelin bei 16 0, 18 1, 18 2 und 20 2 wurden daher statistisch nicht abgesichert Der Vergleich der Fettsäuremuster der Phosphatidhauptfraktion Phosphatidylcholin normaler und leukämischer Lymphozyten ergab signifikante Veränderungen bei Palmitin-, Stearin- und Ölsäure Da schon im Normalfall die Funk-

tion der einzelnen Fettsäuren unklar ist, kann eine Interpretation dieser Abweichungen nicht erfolgen oder müsste zwangsläufig spekulativ sein

Zusammenfassung

Vergleichende Untersuchungen an normalen und leukämischen Leukozyten ergaben keine signifikanten Veränderungen des Gesamtlipidgehalts Lymphozyten von reifzelliger lymphatischer Leukämie enthielten im Vergleich mit normalen Lymphozyten geringere Anteile Triglycerid und Sphingomyelin Das Lezithin leukämischer Lymphozyten enthielt mehr Palmitin und Ölsäure und weniger Stearinsäure als die gleiche Phosphatidfraktion normaler Lymphozyten Die Lipidverteilung normaler und leukämischer Granulozyten war nicht signifikant unterschiedlich

Literatur

1 Bergelson L. D, Dyatlovitskaya, E. V, Torkhovskaya, T I., Sorokina, I B., and Gorkova, N E Phospholipid composition of membranes in the tumor cells Biochim biophys. Acta *210* 287 (1970)

2 Beukers H, Veltkamp, W A., and Hooghwinkel, G J M., A method for the determination of the molecular distribution of free and esterified cholesterol in serum by thinlayer chromatography Clin chem Acta *25* 403 (1969)

3 Fu Chuan Chao, Eng, L. F., and Griffin A Compositional differences of lipid in transformed hamster fibroblasts Biochim biophys Acta *260* 197 (1972)

4 Gottfried E. L. Lipid patterns of leukocytes in health and disease Semin Hemat *9* 241 (1972)

5 Gottfried E. L. Lipid patterns in human leukocytes maintained in long term culture J Lipid Res. *12* 531 (1972)

6 Hakomori, S I The dynamic structure of cell membrane (Springer, Berlin 1971)

7 Jaoe, J Kunze, D und Olthoff D Unsere Erfahrungen mit einer dünnschichtchromatographischen Routinemethode zur Bestimmung der Lipide im Serum Z. med Labortechn. *11* 154 (1970)

8 Kim, H S, Suzuki M., and O'Neal, R Leukocyte lipids of human blood Am J clin Path *43* 314 (1967)

9 Lowry, O H, Rosebrough, N J, Farr A. L., and Randell, R J Protein measurement with the Folin phenol reagent J biol Chem *193* 265 (1961)

10 Martin J B and Doty D M Estimation of inorganic phosphate with an extraction procedure Analyt. Chem. *21* 965 (1949)

11 Morrison W R and Smith, L. M., Preparation of fatty acid methylesters and dimethylacetals from lipids with boron fluoride methanol J Lipid Res *5* 600 (1964)

12 NISHIMURA, K, ODAKA T, and NAGAI, Y Alteration of the glycolipid composition of spleen in Friend leukemia virus infected mice Jap J exp Med *42* 177 (1972)
13 OTTO, F und SCHMID, D Lymphozytenisolierung aus dem Blut des Menschen und der Tiere Blut *21* 1 (1970)
14 OTTO, F Granulozytenisolierung aus dem Blut des Menschen und der Tiere Blut *21* 290 (1970)
15 SCHWANDT, P, BIRK, J und ERHART, H Leukocytenlipide bei Hamoblastosen Klin Wschr *46* 687 (1968)
16 SCHWANDT, P, BIRK, J und ERHART, H Phospholipidzusammensetzung in leukamischen Leukozyten Klin Wschr *47* 111 (1969)
17 WALLACH, D F H The plasma membrane dynamic perspective genetics and pathology (Springer, Berlin 1972)
18 WOOD, R and FALCH, J Lipids of cultured hepatoma cells I Effect of serum lipid levels on cell and media lipids II Effect of media lipids on cellular phospholipids Lipids *8* 690 702 (1973)
19 WOOD, R and HARLOW, R D Tumor lipids structural analyses of phospholipids Archs Biochem Biophys *141* 183 (1970)
20 WOOD, R, ANDERSON, N G., and SWARTZENDRUBER, D C. Tumor lipids characterization of the lipids isolated from membranous material Archs Biochem Biophys *141* 190 (1970)
21 ZÖLLNER N und EBERHAGEN, D Untersuchung und Bestimmung der Lipoide im Blut (Springer, Berlin 1965)

Frau Dr R BLEIBER, I Medizinische Klinik Humboldt Universität Schumannstrasse 20 *DDR 104 Berlin* (DDR)

Acta haemat 55 89–94 (1976)

Relationship between the Concentration of Neutrophils and Monocytes in Venous Blood

BARBARA J BAIN and S N WICKRAMASINGHE

Department of Haematology and MRC Experimental Haematology Unit, [redacted] Mary s Hospital Medical School, University of London, London

Key Words Chronic granulocytic leukaemia Exercise Marginated leucocytes Monocytes Myeloproliferative disorders Neutrophils Pregnancy

Abstract A striking linear relationship was found between the logarithms of the medians of the circulating neutrophil (granulocyte plus metamyelocyte) counts and the monocyte counts in 5 groups of individuals studied. These groups consisted of (1) 100 normal adult males, (2) 100 normal non pregnant females, (3) 50 females in the last trimester of pregnancy (4) 15 patients with a persistent neutrophil leucocytosis and (5) 20 patients with polycythaemia rubra vera, essential thrombocythaemia or myelofibrosis. A different relationship appeared to exist between the circulating neutrophil and monocyte counts in a group of 16 patients with chronic granulocytic leukaemia

In a recent study, TWOMEY *et al* [7] found a linear relationship between the logarithm of the concentration of circulating neutrophils and the logarithm of the concentration of circulating monocytes in a group of individuals consisting of 40 normal adults and 22 patients with aplastic anaemia This observation is of considerable theoretical interest as it supports other data indicating a close relationship between neutrophil granulocytopoiesis and monocytopoiesis Thus several studies have shown that a single progenitor cell can give rise to many monocytes, macrophages and neutrophils when grown in a suitable semi-solid medium in the presence of colony-stimulating factor (CSF) Furthermore, both monocytes and macrophages have been shown to be potent sources of CSF The present study was undertaken to determine the relationship, if any, between the concentration of circulating neutrophils and monocytes in individuals with an elevated neutrophil count. This information would be of import

ance in formulating any hypotheses regarding the mechanism regulating human neutrophil granulocytopoiesis and monocytopoiesis At present, the regulation of both these cell renewal systems is incompletely understood

Patients and Methods

Six groups of individuals were studied These consisted of (1) 100 normal adult males, (2) 100 normal adult females (mainly pre menopausal) (3) 50 females during the last trimester of pregnancy, (4) 15 patients with a reactive neutrophil leucocytosis in whom the neutrophil count had been elevated for a minimum of 7 days (5) 16 patients with chronic granulocytic leukaemia (Ph¹ positive) and (6) a group made up of 10 patients with polycythaemia rubra vera, 5 patients with essential thrombocythaemia and 5 patients with myelofibrosis The diagnoses in the patients with a reactive neutrophil leucocytosis included pyogenic abscesses, bronchopneumonia Crohn s disease and ulcerative colitis The data on the 100 normal males and 100 non pregnant females were taken from an earlier study of the normal neutrophil count, in which a correlation was observed between the circulating neutrophil and monocyte counts [2]

The concentrations of circulating neutrophils neutrophil metamyelocytes and monocytes were calculated from the total nucleated cell count determined on a Coulter counter, model S and a 500-cell differential leucocyte count Only a single sample of venous blood was studied per individual, except in one healthy pre-menopausal adult female In the latter, 80 samples were studied at regular intervals over an 8 month period Except in this subject in whom all the blood counts were performed between 9 00 a m and 10 00 a m after a 30-min period of rest, the counts were performed at any time between 9 00 a m and 5 00 p m, without a preceding period of rest

Pre and immediate post-exercise venous blood samples were studied in 11 healthy adults (8 males and 3 females) who were exercised on a bicycle ergometer The males were exercised for 10 min with a work load of 138 W and the females for 6 min with a work load of 69 W The concentration of various cell types in these samples were calculated from the total nucleated cell counts and 2 000-cell differential counts The post exercise cell counts were corrected for changes induced by haemoconcentration, assuming that the degree of haemoconcentration is proportional to the exercise induced change in the haemoglobin concentration

Results and Discussion

A highly significant correlation was found between the logarithm of the concentration of circulating neutrophil granulocytes plus metamyelocytes (log N) and the logarithm of the concentration of circulating monocytes (log M) in both the normal adult males (r = 0 39, m = 0 40,

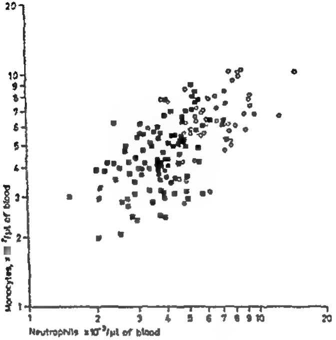


Fig 1 Relationship between the concentration of neutrophil granulocytes plus metamyelocytes and the concentration of monocytes in the circulation of 100 healthy non pregnant females (■) and 30 females in the last trimester of pregnancy (○)

$p<0.001$) and the normal adult females ($r = 0.45$, $m = 0.49$, $p<0.001$) A similar correlation was also seen in the group of pregnant females ($r = 0.41$, $m = 0.45$, $p<0.001$) and the regression line for this group was virtually identical to that for the group of non-pregnant females (fig 1) Although all the above-mentioned correlations reached a high degree of statistical significance, the correlations were quite weak; only 15–20% of the observed variation in log M can be accounted for by variation in log N and vice versa This poor correlation was not entirely due to person to-person variations in the relationship between log M and log N or to diurnal variations in cell counts as the correlation between log N and log M in the samples of blood taken between 9 00 a m. and 10 00 a m on 80 different days from a single pre-menopausal woman ($r = 0.31$) was not significantly different from that in the entire group of non pregnant fem-

Table I. Pre- and post-exercise leucocyte counts in 11 normal subjects

Cell type	Median cell count $\times 10^{-6}$/l		Post-exercise/pre-exercise		Significance of exercise-induced change p
	pre-exercise	post-exercise[1]	range	median	
Neutrophil granulocytes + metamyelocytes	3,306	3,915	1.08–1.41	1.18	<0.001
Lymphocytes	2,094	4,006	1.67–2.51	1.91	<0.001
Monocytes	457	778	1.15–2.54	1.70	<0.001
Eosinophil granulocytes	105	151	1.02–2.17	1.45	<0.01
Basophil granulocytes[2]	35	48	0.63–3.09	1.39	<0.05

[1] Corrected for post-exercise haemoconcentration.
[2] A post exercise increase was found in 9 of the 11 subjects.

ales. As CARTWRIGHT *et al.* [4] have shown that the fraction of the total blood granulocyte pool which circulates may vary between 16 and 99% in resting, normal individuals, it is possible that the weak correlations observed in this study are partly caused by a poor correlation between the sizes of the circulating and total blood granulocyte pools. It is also possible that one or more factors which are independent of the size of the total blood neutrophil or monocyte pool (e.g. oestrogen and progesterone levels in females) affect log N and log M to different extents.

Vigorous exercise, which mobilises cells from the marginated to the circulating leucocyte pool [1], causes a greater increase in the concentration of circulating monocytes than in the concentration of circulating neutrophil granulocytes plus metamyelocytes (table I), indicating that changes in the equilibrium between the marginated and the circulating leucocyte pools affect the correlation between log N and log M. It is of interest that the results shown in table I provide evidence for the existence of fairly large marginated pools of eosinophil and basophil granulocytes. A post-exercise increase in eosinophil and basophil counts was also observed by BELSKY *et al.* [3].

There was no correlation between log N and log M in the groups with (1) a reactive neutrophil leucocytosis (r = 0.15, 95% confidence limits, –0.39 to +0.61), (2) polycythaemia vera, myelofibrosis or essential thrombocythaemia (r = –0.11, 95% confidence limits, –0.53 to +0.35)

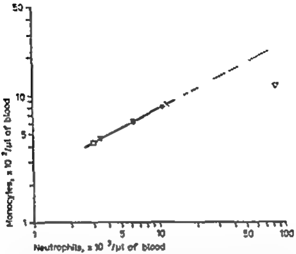


Fig 2 Relationship between the median values for the concentration of circulating neutrophil granulocytes plus metamyelocytes and the concentration of circulating monocytes in the various groups studied. □ = 100 normal males, ■ = 100 non-pregnant, normal females, ■ = 50 pregnant females, ● = 15 patients with a reactive neutrophil leucocytosis, ▽ = 16 patients with chronic granulocytic leukaemia, × = 20 patients with other chronic myeloproliferative disorders, ▼ = a single normal, non pregnant female studied on 80 different days, over an 8 month period

and (3) chronic granulocytic leukaemia ($r = -0.12$, 95% confidence limits, -0.28 to $+0.37$) However, the relationship between log N and log M which exists in normal adults is found to apply to the first two of these groups, if the median values for the concentration of circulating neutrophils (granulocytes plus metamyelocytes) and monocytes are considered With the exception of the group of patients with chronic granulocytic leukaemia, there was a striking linear relationship between the logarithms of these median values (fig 2) This implies that in reactive leucocytosis and myeloproliferative disorders (other than chronic granulocytic leukaemia), a weak correlation does exist between log N and log M but that this correlation may not become evident until much larger groups of patients are studied

In chronic granulocytic leukaemia, the relationship between log N and log M is altered Whereas the median neutrophil count is increased 28-fold in comparison with normal males, the median monocyte count is in-

creased only 3 fold (fig 2) The explanation for the 'abnormal' relationship between log N and log M which occurs in chronic granulocytic leukaemia is uncertain Three possible explanations are (1) that the *in vitro* colony-forming cells derived from the Ph¹-positive multipotent haemopoietic stem cells respond abnormally to the various regulatory mechanisms which govern the production of neutrophils and mononuclear phagocytes, (2) that these regulatory mechanisms are disturbed in chronic granulocytic leukaemia and (3) that the linear relationship between log N and log M does not hold when the circulating neutrophil count is very high It must also be mentioned that disturbances in the normal relationship between log N and log M are not confined to patients with chronic granulocytic leukaemia It has been known for a considerable time that the circulating neutrophil and monocyte counts do not change synchronously during the development or resolution of a neutrophil leucocytosis [5], nor do they in cyclical neutropenia [6]

Acknowledgements We are grateful to Dr D A G GALTON for valuable comments during the preparation of this manuscript

References

1 ATHENS, J W, RAAB, S O, HAAB, O P, MAUER, A M, ASHENBRUCKER H CARTWRIGHT, G E., and WINTROBE, M M Leukokinetic studies III The distribution of granulocytes in the blood of normal subjects J clin Invest *40* 159 (1961)
2 BAIN, B J and ENGLAND, J M Normal haematological values sex difference in neutrophil count Br med J *i* 306 (1975)
3 BELSKY, J L., ISHIMARU, T, OHASHI, T, ROBERTSON, T L., and TANIGUCHI S Leucocyte response to exercise in atomic bomb survivors Radiat. Res *50* 699 (1972)
4 CARTWRIGHT, G E., ATHENS, J W and WINTROBE, M M The kinetics of granulopoiesis in normal man Blood *24* 780 (1964)
5 HICKLING, R A The monocytes in pneumonia Archs intern Med *40* 594 (1927)
6 MOORE, M A S, SPITZER G, METCALF, D., and PENINGTON, D G Monocyte production of colony stimulating factor in familial cyclic neutropenia Br J Haemat *27* 47 (1974)
7 TWOMEY, J J, DOUGLASS, C. C., and SHARKEY, O., jr The monocytopenia of aplastic anemia Blood *41* 187 (1973)

Dr S N WICKRAMASINGHE, Department of Haematology, St Mary's Hospital Medical School *London W2 1PG* (England)

Acta haemat. 55 95–98 (1976)

Increased Proportion of B Lymphocytes in Human Tonsils and Appendices

C. ALEXOPOULOS, A. G. PAPAYANNIS and C. GARDIKAS

Professional Medical Unit, Evangelismos Hospital, Athens

Key Words Appendix Lymphocytes Tonsils

Abstract The percentage of T and B lymphocytes was studied in human tonsils and appendices. An increased percentage of B lymphocytes, 59.1% in tonsils and 54.5% in appendices, was found, whilst the percentage of T lymphocytes was [illegible] 6% and 27.9%, respectively. In the peripheral blood the corresponding figures were 12.5% for B cells and 50% for T cells.

It has been recently shown that there are two major populations of lymphocytes [10, 13]: thymus-derived (or T) lymphocytes, involved mainly with cell mediated immunity and bursa-derived (or B) lymphocytes mediating humoral immunity. The reported percentage for T lymphocytes in the peripheral blood varies normally from 40 to 58% [4, 6] while that for B lymphocytes varies from 13 to 34% [9, 13]. In the spleen and the lymph nodes the percentage of T lymphocytes varies from 20 to 36.5% [2, 11] and from [illegible] to 60% [3, 11] respectively, while that of B lymphocytes varies from 45 to 58% in the spleen [2, 5, 12] and from 21 to 31% in the lymph nodes [8, 11].

In view of the lack of information about T and B cell populations in human tonsils [8] and appendix we thought it worthwhile to study the distribution of T and B lymphocytes in these organs.

Material and Methods

Lymphocytes. Lymphocytes were obtained from the peripheral blood of 10 normal subjects, from tonsils of 10 patients undergoing tonsillectomy and from append

ices of 10 patients with appendectomy In 2 cases, normal appendices were removed during hysterectomy

Lymphocytes were separated from the peripheral blood by means of Ficoll Hypaque Lymphocytes from tonsils and appendices were isolated by finely mincing the organs in a small volume of Medium 199 and removing the debris by filtration through fine steel mesh

Surface immunoglobulins A direct immunofluorescence method [11] was used by means of fluorescein conjugated polyvalent antiserum (anti IgG $\varkappa$ and λ, Hyland)

EAC rosettes AB Rh+ human red blood cells (HRBC) from heparinized whole blood were washed thrice in normal saline, 2 ml of a 5% HRBC suspension in Hanks' balance salt solution (HBSS) were mixed with equal volume of inactivated rabbit anti HRBC serum (prepared by injecting AB Rh+ HRBC in a rabbit) diluted 1 1000 and the mixture incubated at 37 °C for 1 h The cells were washed twice in veronal buffer saline (VBS) Then 2 ml of fresh mouse serum (as a source of complement) diluted 1 10 in VBS was added to 2 ml of cell suspension and incubated at 37 °C for 1 h The red cells were finally washed twice and adjusted to 0 5% suspension in HBSS (C-coated HRBC)

Of the lymphocyte suspension 0 4 ml containing 1×10^6 cells were mixed with 0 4 ml of the 0 5% C-coated HRBC suspension and rotated gently for 30 min at room temperature One drop was mounted onto a glass slide, covered by a coverslip and the percentage of lymphocytes binding three or more red cells was determined after counting 300 lymphocytes

Spontaneous rosette formation with sheep red blood cells was used to determine T lymphocytes [7]

Results

The results are summarized in the tables I and II The mean percentage of T lymphocytes in the peripheral blood of normal subjects was 50% compared to 23 6 and 27 9% of the tonsils and appendices, respectively The mean percentage of B lymphocytes in the peripheral blood was 12 5% while it was significantly higher in the tonsils (59 1%) and appendices (54 5%) There was no significant difference between the results obtained by the EAC-rosettes and those obtained by the surface-bound immunoglobulins

Discussion

The present observations indicate that human tonsils and appendix contain a large proportion of B lymphocytes, much higher than the peripheral blood On the other hand, the percentage of T lymphocytes in the above organs is significantly lower than in the blood Two possible ex

Table I Percentage of B lymphocytes

	Peripheral blood		Tonsil		Appendix	
	EAC	Ig	EAC	Ig	EAC	Ig
	9	7	54	56	55	57
	10	9	56	57	48	52
	12	12	50	55	48	50
	11	12	58	60	50	50
	9	11	59	62	50	55
	9	10	55	[illegible]	47	50
	20	9	52	50	49	54
	18	20	60	64	50	52
	14	19	58	[illegible]	68	65
	10	16	65	68	58	60
Mean	12.2	12.5	56.7	59.1	51.5	54.5

Table II Percentage of T lymphocytes

	Peripheral blood	Tonsil	Appendix
	45	28	27
	60	26	31
	54	28	25
	50	23	25
	52	20	24
	[illegible]	23	37
	52	27	34
	47	22	[illegible]
	46	21	[illegible]
	46	18	23
Mean	40	23.6	27.9

planations may be offered for the present findings (1) the presence of chronic inflammation may be the cause of regional accumulation of B lymphocytes although in two appendices where no inflammation existed the percentage of B cells was equally high, or (2) the high percentage of B lymphocytes found in tonsils and appendices could support the hypothesis that the lymphoid tissue associated with the gastrointestinal system may constitute the equivalent of the bursa in mammals

References

1 AISENBERG, A C and BLOCH, K J Immunoglobulins on the surface of neoplastic lymphocytes New Engl J Med *287* 272 (1972)

2 HABESHAW, J A and STUART, A E T and B cells in human spleens. Lancet *I* 1164 (1974)

3 JAFFE, E S, SHEVACH, E M, FRANK, M M, BERARD, C W, and GREEN, I Nodular lymphoma Evidence for origin from follicular B lymphocytes New Engl J Med *290* 813 (1974)

4 JONDAL, M, HOLM G, and WIGZELL, H Surface markers on human T and B lymphocytes J exp Med *136* 207 (1972)

5 KAUR, J, CATOVSKY, D, SPIERS, A S D, and GALTON, D A G Increase of T lymphocytes in the spleen in chronic granulocytic leukaemia Lancet *i* 834 (1974)

6 LAY, W H, MENDES, N F, BIANCO, C, and NUSSENZWEIG, V Binding of sheep red blood cells to a large population of human lymphocytes Nature, Lond *230* 531 (1971)

7 PAPAMICHAIL, M, HOLBOROW, E J, KEITH H I, and CURREY, H L F Subpopulations of human peripheral blood lymphocytes distinguished by combined rosette formation and membrane immunofluorescence Lancet *ii* 64 (1972)

8 PINCUS S, BIANCO, C, and NUSSENZWEIG, V Increased proportion of complement receptor lymphocytes in the peripheral blood of patients with chronic lymphocytic leukemia Blood *40* 303 (1972)

9 PREUD HOMME, J L and SELIGMANN, M Surface bound immunoglobulins as a cell marker in human lymphoproliferative diseases Blood *40* 777 (1972)

10 RAFF, M C T and B lymphocytes in mice studied by using antisera against surface antigenic markers Am J Path *65* 467 (1971)

11 SILVEIRA, N P A, MENDES N F, and TOLNAI, M E A Tissue localization of two populations of human lymphocytes distinguished by membrane receptors J Immun *108* 1456 (1972)

12 VISAKORPI R and REPO, H T lymphocytes in human spleen Lancet *ii* 740 (1973)

13 WILSON, J D and NOSSAL, G J V Identification of human T and B lymphocytes in normal peripheral blood and in chronic lymphocytic leukaemia Lancet *ii* 788 (1971)

Prof C GARDIKAS, Professorial Medical Unit Evangelismos Hospital *Athens 140* (Greece)

Acta haemat. 55: 99–108 (1976)

Activity of Inhibitors on Plasmatic Erythropoietin and on the Renal and Splenic Erythropoietic Factors in the Rabbit

ELVIO GIOVANNINI

Institute of General Biology, Faculty of Medicine and Surgery, University of Perugia, Perugia

Key Words: Cleland's reagent · Erythropoiesis · Erythropoietin · Koshland's reagent · Renal erythropoietic factor · Splenic erythropoietic factor

Abstract. The author has studied the action of Cleland's reagent and Koshland's reagent on the erythropoietic factors present in the kidneys and the spleen of normal rabbits, in the plasma of bled rabbits and in the mixtures obtained through incubation of normal plasma with kidney and spleen homogenates. The results show that Cleland's reagent inhibits the activity of the renal and splenic erythro-stimulant factors, whereas it has no effect upon the erythropoietin in anaemic plasma and in plasma-kidney and plasma-spleen incubation mixtures. The activity of erythropoietin, on the other hand, is inhibited by Koshland's reagent, which has no influence on the renal and splenic erythro-stimulant factors.

LEWIS *et al.* [5] have identified the presence of erythro-stimulant factors in the urine of anaemic patients; in particular, in addition to erythropoietin, an erythropoietic factor has also been found, possessing certain characteristics similar to those of the renal factor studied by CONTRERA and GORDON [1] and by ZANJANI *et al.* [13], since it gives rise to the production of erythropoietin if incubated with serum of normal rabbits.

LEWIS *et al.* [6] later studied the effects of Cleland's reagent (2,3-dihydroxy-1-4-dithiolbutane or dithiothreitol, DTT), known for its capacity to hinder the oxidation of the –SH groups, on the two different erythro-stimulant factors in urine. They showed that DTT was capable of completely inactivating the erythropoietic factor content in urine, whereas it had no effect on the erythropoietin, even in very high doses. It therefore seems that the activity of the erythropoietic factor is connected with the presence of –SH groups, though these are not necessary for the bio-

logical activity of erythropoietin, as is also shown by the studies of GOLDWASSER and KUNG [4]. Moreover, also LOWY and KEIGHLEY [11] state specifically that DTT does not inactivate erythropoietin.

On the other hand, these last authors have shown [11] that erythropoietin is inactivated by Koshland's reagent (2-hydroxy-5-nitrobenzyl-bromide), which reacts with the tryptophane in the protein chains, forming substitution products with the indolic ring and leaving the peptide bonds unharmed.

The object of the present studies was to verify whether DTT, known for its capacity to inhibit the erythropoietinogenic factor present in the urine of anaemic patients [6], was equally capable of inactivating the renal erythropoietic factor and the erythropoietic factor present in the spleen of the rabbit, which it also considered to be of renal origin [8]. Another object of the studies was to verify whether Koshland's reagent, known for its capacity to inhibit erythropoietin, was equally capable of inhibiting the activity of the plasma-kidney and plasma-spleen incubation products, which probably contain erythropoietin [9]. A final object was to establish if the systems considered responded differently to the same inhibitor, in order to derive further confirmation of the hypothesis of the different nature of the factors contained in the kidney and in the spleen, compared with those contained in anaemic plasma or in plasma-kidney and plasma-spleen incubation products.

Material and Methods

Extraction of the renal and splenic factors. The light mitochondrial and microsomal fractions of the kidney and the mitochondrial fraction of the spleen, containing the erythro-stimulant factors were isolated from normal rabbits [3]. The hypotonic extracts of these subcellular fractions were then subjected to fractionation with ammonium sulphate in concentrations of 30 and 70% saturation, in accordance with the first stage of the technique followed by WONG et al. [12]. The two fractions thus obtained were suspended again in 20 ml of bi-distilled water and subjected to dialysis in order to eliminate the ammonium sulphate. On evaporating, in vacuum, the dialysed product relative to the fractions obtained from the kidneys or from the spleen with the second precipitation – carried out, as has been stated, with ammonium sulphate at 70% saturation – a residual material was obtained, weighing on the average, 48 mg (kidney) and 9.8 mg (spleen), with very small variations in these values.

Extraction of the plasmatic factors. With similar aims, an attempt was made to obtain, in sufficiently concentrated form, both the erythropoietin contained in the plasma from bled rabbits (20 ml/kg of body weight), 24 h after bleeding, and that

present in the incubation mixtures prepared with plasma and kidney or spleen homogenates from normal rabbits [9]. For these experiments a technique similar to that described by LOWY and BORSOOK [10] was followed, in particular, both the anaemic plasma relative to each donor (50 ml) and the above-mentioned incubation products (50 ml of normal plasma mixed with the hypotonic homogenate of a kidney or a spleen) were heated in boiling water and then cooled and centrifuged. In order to obtain a more effective precipitation of the thermo-labile protein substances present in the materials studied 1–1.5 g of NaCl was added before heating since the acidification of the material carried out according to LOWY and BORSOOK's [10] technique might have given rise in the presence of spleen homogenate, to the inactivation of the erythropoietin in the incubation mixtures obtained with that organ. In the course of previous studies [9] the splenic tissue was found to possess a factor capable of inactivating or degrading erythropoietin in an acid environment. The supernatant obtained by the above mentioned methods was then subjected to fractionation by means of absolute ethanol and the fraction precipitated with 60–80% concentrations of ethanol was then dried in vacuum and weighed. The average weight of these fractions was 21.2 mg for the anaemic plasma, 23.5 mg for the plasma spleen incubation material and 30.1 mg for the plasma kidney incubation material.

The active fractions of renal, splenic or plasmatic origin obtained by the methods described above were then subjected to the action of certain inactivating substances (DTT and Koshland's reagent) in order to verify whether they were capable of suppressing the erythro-stimulant activity. These experiments were performed in conjunction with others as a control in order to verify the existence of an activity in the preparations used.

Cleland's reagent and renal splenic erythropoietic factors. The active material corresponding to the kidneys of a donor and prepared by the methods described above was incubated for 30 min with 5 mg of DTT at 37 °C [6]. Preliminary experiments carried out by using scalar doses of this reagent had shown that a dose of 5 mg was sufficient to inactivate the erythropoietic factor contained in the kidneys of a normal animal. Similarly the active fraction corresponding to a spleen was treated with 1 mg of DTT – the dose calculated in relation to the average weight of the residual material obtained by evaporating the fraction in vacuum (9.8 mg) which is found to be about one fifth of that of the material obtained with the same methods from kidney extracts (49 mg).

Koshland's reagent and plasmatic erythro-stimulant factors. The preparations rich in erythropoietin obtained from the anaemic plasma or from the kidney plasma and spleen plasma incubation mixtures by the methods already described, were dried in vacuum and were then dissolved in bi-distilled water in a concentration of 0.1%. To these preparations an equal quantity of Koshland's reagent dissolved in 0.5 ml of acetone was added. The reaction mixture was then kept at 23 °C for 1 h and subjected to dialysis against bi-distilled water in order to eliminate the acetone and the reagent [11].

The reverse use of the reagents on the erythropoietic factors. In the course of preliminary tests, Koshland's reagent was found to have absolutely no effect on the renal and splenic erythropoietic factors, when added to the active fractions in a quantity equal to their average weight. Other tests were therefore carried out using this reagent in very high doses, equal to 10 times the average weight of the fractions

(480 mg in the inactivation tests carried out on the renal material and 98 mg in those carried out on the splenic material). These doses were dissolved in 10 ml and in 2 ml of acetone, respectively, and then added to the renal or splenic material under examination. The incubation and the subsequent dialysis were performed with the usual technique.

Similar inactivation tests were then carried out by incubating DTT, known for its inability to inhibit erythropoietin [6, 11] with active fractions prepared from kidney plasma and spleen plasma mixtures, also making use of fractions containing erythropoietin, since they were obtained from anaemic plasma. Also in these experiments, high doses of the reagent were used (20–30 mg of DTT), being proportionally equal to 10 times those sufficient to inhibit renal or splenic active fractions having the same weight, since preliminary tests, carried out by using such doses (2–3 mg of DTT) had given negative results.

With the preparations thus obtained, various experiments were performed. First of all, the erythro-stimulant activity of the various extraction materials was studied. A test was then made to verify whether DTT and Koshland's reagent were capable of suppressing the erythro-stimulant activity present in these materials.

These activities were assessed by studying the variations in the reticulocyte pattern in the peripheral blood of rigidly selected rabbits, later treated with the various preparations under examination by following the criteria already described in previous works [2, 3]. In particular, in the extraction tests for the various erythro-stimulant factors, use was made of groups of 6 normal or bled donors, from which organs or plasma were removed and, at the same time, of groups of 6 receivers, each of which was to be treated with the material obtained from a single donor. On the other hand, in the inactivation tests including also the control experiments, use was made of groups of 12 donors, killed two at a time. The active fractions obtained from each pair of animals were combined into a single complex, half of which was incubated with the inactivator under study and the remaining half used in the control experiment. Also in these tests, groups of 6 receivers for each preparation to be tested were used.

The material to be administered to each animal was, in every case, brought up to a volume of 40 ml by means of bi-distilled water, made isotonic by adding NaCl, divided into two doses to be given by intraperitoneal injection at an interval of 24 h and kept at –20 °C until use.

Results

The results obtained by the treatment with fractions of subcellular extracts of kidney and of spleen (table I) show that in both organs the fractions sedimented with ammonium sulphate at 30–70% saturation (groups 1B, D) produce a highly significant increase in the reticulocyte content, whereas those sedimented with 30% ammonium sulphate (groups 1A, C) do not produce any significant variation in the number of circulating reticulocytes. These active fractions of subcellular extracts of kidney and of

ble I Variations of the reticulocyte level[1] with time in rabbits treated with material sedimented usi $(NH_4)_2SO_4$ at 30% (groups 1A, C) and at 30–70% (groups 1B, D) saturation from a hypotonic extract light mitochondrial and microsomal fractions of kidney (groups 1A, B) or of the mitochondrial fractic of the spleen (groups 1C, D) of normal donors

	Group 1A		Group 1B		Group 1C		Group 1D	
	mean ± SE	t test	mean ± SE	t-test	mean ± SE	t test	mean ± SE	t tes
itial	2.3 ±0.0931		2.35±0.0957		2.6 ±0.1291		2.35±0.1310	
lays	2.55±0.0957	1.872	3.55±0.1522	6.674[2]	2.75±0.1310	0.8157	3.97±0.1406	8.43
lays	2.15±0.1384	0.8992	3.95±0.1384	9.423[2]	2.7 ±0.1065	0.5976	4.35±0.1747	9.16
lays	2.5 ±0.1506	1.13	3.45±0.1648	5.771[2]	2.82±0.1514	1.105	3.82±0.1301	7.96
lays	2.65±0.1668	1.832	2.55±0.1176	1.322	2.62±0.1352	0.107	2.58±0.1492	1.1

[1] The reticulocyte level is expressed as a reticulocyte/erythrocyte percentage. Mean values ± standa ror and comparison between the averages of the initial and subsequent values by means of Student's t-t (f 10)

[2] Highly significant.

able II Variations of the reticulocyte level[1] with time in rabbits treated with material sedimented usi $(NH_4)_2SO_4$ at 30–70% saturation from a hypotonic extract of the light mitochondrial and microsom ractions of kidney (groups 2A, B) or of the mitochondrial fraction of the spleen (groups 2C, D) of norm donor, given as a control (groups 2A, C) or after further incubation with DTT (groups 2B, D)

	Group 2A (control)		Group 2B		Group 2C (control)		Group 2D	
	mean ± SE	t-test	mean ± SE	t test	mean ± SE	t test	mean ± SE	t te
nitial	2.15±0.1118		2.32±0.1352		2.42±0.1077		2.52±0.1249	
days	3.73±0.1204	9.737[2]	2.57±0.1115	1.427	3.78±0.1301	8.052[2]	2.7 ±0.1592	0.69
days	4.0 ±0.1732	8.974[2]	2.3 ±0.1633	0.0943	4.15±0.1232	10.57[2]	2.63±0.1256	0.6
days	3.55±0.1137	8.967[2]	2.2 ±0.1155	0.6751	3.82±0.1492	7.607[2]	2.62±0.1424	0.5
5 days	2.33±0.0958	1.206	2.25±0.1354	0.3618	2.65±0.1354	1.311	2.7 ±0.1317	0.9

[1, 2] See table II

spleen, if previously incubated with DTT (table II), lose their erythro-stimulant capacity, since – in consequence of this incubation – they are no longer able to produce any significant variation in the reticulocyte pattern in the treated animals (groups 2B, D).

If the same active fractions are incubated with Koshland's reagent

Table III Variations of the reticulocyte level[1] with time in rabbits treated with material sedimented by $(NH_4)_2SO_4$ at 30–70% saturation from a hypotonic extract of the light mitochondrial and microsomal fractions of kidney (groups 3A, B) or of the mitochondrial fraction of the spleen (groups 3C, D) of normal donor, given as a control (groups 3A, C) or after further incubation with Koshland's reagent (groups 3B, D)

	Group 3A (control)		Group 3B		Group 3C (control)		Group 3D	
	mean±SE	t test	mean±SE	t test	mean±SE	t test	mean±SE	t test
Initial	2 68±0 1621		2.2 ±0 1238		2 45±0 1204		2 12±0 1400	
2 days	3 85±0 1432	5 41[2]	3 65±0 1628	7 09[2]	3 6 ±0 1483	6 02[2]	3 62±0 1740	6.71[2]
3 days	4 28±0 1579	7 07[2]	3 92±0 1579	8 57[2]	3 88±0 1579	7 202[2]	3 75±0 1455	8 07[2]
4 days	4 0 ±0 1592	5 811[2]	3 72±0 1778	7 016[2]	3 63±0 1308	6 638[2]	3 48±0 1166	7 46[2]
6 days	2 95±0 1544	1 206	2 37±0 1406	0 9074	2 32±0 1275	0 7411	2 38±0 1352	1.3[illegible]

[1] [2] See table I

Table IV Variations of the reticulocyte level[1] with time in rabbits treated with material obtained by means of heating, centrifugation and fractionation with C_2H_5OH at 60–80% concentration from plasma of donor bled 24 h before (group 4A) or from a kidney-plasma (group 4B) or spleen plasma (group 4C) incubation mixture of normal donors

	Group 4A		Group 4B		Group 4C	
	mean ± SE	t test	mean ± SE	t test	mean ± SE	t test
Initial	2 63±0 1030		2 62±0 1424		2 32±0 1166	
2 days	3 88±0 1400	7 136[2]	3 92±0 1641	5 948[2]	3 55±0 1522	6 415[2]
3 days	4 33±0 1801	8 148[2]	4 33±0 1453	8 407[2]	3 92±0 1470	8 528[2]
4 days	3 72±0 1661	5 541[2]	3 75±0 1708	5 083[2]	3 67±0 1382	7 46[2]
6 days	2 92±0 1887	1 342	2 57±0 1605	0 2331	2 45±0 1310	0 7411

[1] [2] See table I

however, even if it is used in strong doses (table III), they preserve their erythro-stimulant capacity Even after this incubation, they produce a highly significant increase in the reticulocyte pattern (group 3B, D)

The material obtained by means of heating, centrifuging and fractionation with 60–80% ethanol (table IV), whether it comes from plasma of an anaemic donor (group 4A) or from incubation mixtures kidney-normal plasma (group 4B) or spleen-normal plasma (group 4C) produces a highly significant increase of reticulocytes However, in comparison with the two

Table V Variations of the reticulocyte level[1] with time in rabbits treated with material obtained by means of heating, centrifugation and fractionation with C_2H_5OH at 60–80°, concentration from plasma of a donor bled [illegible] h before (groups A, a) and from kidney-plasma (groups B, b) or spleen plasma (groups C, c) incubation mixture relative to a normal donor, given as a control (groups A, B, C) or after further incubation with DTT (groups a, b, c)

	Group 5A (control)		Group 5a	
	mean ± SE	t test	mean ± SE	t test
Initial	2.5 ± 0.1315		2.27 ± 0.1054	
2 days	3.82 ± 0.1470	6.69[3]	4.02 ± 0.1424	9.881[2]
3 days	4.28 ± 0.1447	9.099[2]	4.15 ± 0.1204	11.75[2]
4 days	3.63 ± 0.1229	6.275[3]	3.72 ± 0.1194	9.104[2]
6 days	2.75 ± 0.1335	1.333	2.48 ± 0.1470	1.161
	Group 5B (control)		**Group 5b**	
	mean ± SE	t test	mean ± SE	t test
Initial	2.12 ± 0.1077		2.38 ± 0.1272	
2 days	3.68 ± 0.1579	8.162[2]	3.57 ± 0.1475	6.049[2]
3 days	3.93 ± 0.1282	10.81[2]	3.82 ± 0.1711	6.7[2]
4 days	3.58 ± 0.1514	7.857[2]	3.65 ± 0.1455	6.506[2]
6 days	2.12 ± 0.1166	0	2.27 ± 0.1202	0.6211
	Group 5C (control)		**Group 5c**	
	mean ± SE	t test	mean ± SE	t-test
Initial	2.18 ± 0.1352		2.58 ± [illegible].1249	
2 days	3.65 ± 0.1500	8.28[2]	3.75 ± 0.1565	5.843[2]
3 days	3.85 ± [illegible].1658	7.806[2]	4.22 ± 0.1923	7.156[2]
4 days	3.72 ± 0.1275	8.287[2]	3.57 ± 0.1144	5.844[2]
[illegible] days	2.33 ± 0.1201	0.8295	2.75 ± 0.1360	0.9206

[1, 2] See table I

reagents previously employed, these fractions display an opposite behaviour. If these active fractions are incubated with DTT (table V) before being administered to receiver animals, they preserve their erythro-stimulant capacity (groups 5a–c), since they produce highly significant variations in the reticulocyte pattern, which are exactly like those found in control animals (groups 5A–C). This erythro-stimulant activity persists if

Table VI Variations of the reticulocyte level[1] with time in rabbits treated with material obtained by means of heating centrifugation and fractionation with C_2H_5OH at 60–80°, concentration from plasma of a donor bled 24 h before (groups A, a) and from a kidney plasma (groups B, b) or spleen plasma (groups C, c) incubation mixture relative to a normal donor, given as a control (groups A–C) or after further incubation with Koshland's reagent (groups a–c)

	Group 6A (control)		Group 6a	
	mean ± SE	t test	mean ± SE	t test
Initial	2 05 ± 0.1232		2 15 ± 0.1283	
2 days	3 57 ± 0 1406	8 133[2]	2 0 ± 0 1211	0 8496
3 days	3 82 ± 0 1352	9 68[2]	2.32 ± 0 1327	0 9206
4 days	3 38 ± 0 1600	6 857[2]	2.27 ± 0 0954	0.75
6 days	2 27 ± 0 1453	1 155	2.28 ± 0 1249	0 7256
	Group 6B (control)		Group 6b	
	mean ± SE	t test	mean ± SE	t test
Initial	2 45 ± 0 1148		2 7 ± 0 1366	
2 days	3 67 ± 0 143	6 656[2]	2 92 ± 0 1778	0.9812
3 days	4 13 ± 0 152	8 821[2]	2.92 ± 0 2056	0 8913
4 days	3 5 ± 0 1265	6 148[2]	2 73 ± 0 1308	0.1586
6 days	2.65 ± 0 1384	1 112	2 85 ± 0 1586	0 7165
	Group 6C (control)		Group 6c	
	mean ± SE	t test	mean ± SE	t test
Initial	2 33 ± 0 1256		2 72 ± 0 1514	
2 days	3.35 ± 0 1408	5 936[2]	2 53 ± 0 152	0 8856
3 days	3 82 ± 0 1376	8 536[2]	2 63 ± 0 1453	0 4289
4 days	3.27 ± 0 1308	5 736[2]	2 6 ± 0 1183	0 6244
6 days	2.55 ± 0 1384	1 712	2.87 ± 0 1333	0 7435

[1, 2] See table I

the DTT is used in much higher doses than those sufficient to inhibit the preparations obtained from the kidney and from the spleen

The fractions obtained from the anaemic plasma and from the kidney-plasma and spleen plasma incubation mixtures lose their erythro stimulant capacity if they are incubated with Koshland's reagent before being used (table VI) In fact, in these experimental conditions they do not produce any significant variation in the reticulocyte pattern (groups 6a–c)

Discussion

Assessing the results obtained by means of treatment with protein fractions of renal or splenic subcellular extracts, it may be stated that, if the erythro-stimulant efficacy of the treatment carried out with the fraction of renal extract sedimented with ammonium sulphate at 30–70%, saturation could also have been taken for granted to a certain extent – in consideration of the results already obtained by Wong *et al* [12] in the rat – the similar activity present in the splenic fraction obtained with the same technique nevertheless constitutes a new element, since the role of the spleen in the erythropoiesis of the rabbit has been explained only by the studies carried out in this Institute in recent years [7, 8]

On the basis of the results obtained by administering renal or splenic fractions rich in erythropoietic factors, after incubation with DTT, it appears that this reagent, in the experimental conditions adopted, inactivates the factor previously identified in the renal tissue of the rabbit [3, 8] It is therefore probable that it is to be identified with that described by Lewis *et al* [5, 6] in the urine of anaemic patients a hypothesis also suggested by the results of other recent studies [2] The fact that in the same experimental conditions the DTT also inactivates the factor present in the spleen of the rabbit appears to be in agreement with the results of previous works, which show that there occurs, in the spleen, an accumulation of an erythropoietic factor produced and released into the circulation by the kidney [7, 8]

The hypothesis that the erythropoietic factors contained in the kidney and in the spleen are of the same nature is also strengthened by the fact that the erythro stimulant activity of both is inhibited by the DTT but not by Koshland s reagent These factors cannot be identified with erythropoietin, since this is not inactivated by the DTT [6, 11] whereas Koshland s reagent is an effective inhibitor of it [11] Moreover, the fact that the active fractions obtained from kidney plasma and spleen plasma incubation mixtures, like those obtained from anaemic plasma, do not lose their erythro-stimulant activity if treated with DTT, whereas they lose it completely if treated with Koshland s reagent, shows that these mixtures, like anaemic plasma do not contain the same erythropoietic factors as those present in the kidney and in the spleen, but contain erythropoietin

All these data therefore show that the erythropoietic factors present in the kidney and in the spleen differ from the erythropoietin to be found in anaemic plasma and in kidney normal plasma and spleen normal plasma

incubation mixtures, not only in their molecular weight, as recently demonstrated [2], but also in other physicochemical characteristics

References

1 CONTRERA, J F and GORDON, A S Erythropoietin production by a particulate fraction of rat kidney Science, N Y *152* 653–654 (1966)

2 GIOVANNINI E and BOCCHINI, V Different molecular weight of the plasmatic erythropoietin compared with the renal and splenic erythropoietic feators in the rabbit Acta haemat *53* 75–81 (1975)

3 GIOVANNINI, E and ROSI, G The subcellular localization of the erythro-stimulant factors present in the kidney and spleen of the rabbit Br J Haemat *29* 129–135 (1975)

4 GOLDWASSER, E and KUNG, K H Progress in the purification of erythropoietin Ann N Y Acad Sci *149* 49–53 (1968)

5 LEWIS J P, ALFORD D A, NEAL, W A, MOORES, R R, WELCH E T, GARDNER E jr, WRIGHT, C S, and SMITH, L L Erythropoiesis stimulating factors Scand J Haematol *8* 200–209 (1971)

6 LEWIS, J P, WELCH, E T, NEAL, W A, MOORES, R R, LEWIS, W E WRIGHT, C S, SMITH, L L, and DUBOSE, C M jr *The selective inactivation of an ESF generating factor (EGF) in the presence of erythropoietin (ESF)* Proc Soc exp Biol Med *137* 783–785 (1971)

7 LIOTTI, F S e GIOVANNINI, E *Rapporti tra rene e milza nel controllo dell eritropoiesi* Haematologica *54* 351–370 (1969)

8 LIOTTI, F S e GIOVANNINI, E Studi e ricerche in tema di rapporti tra rene e *milza nel controllo dell'eritropoiesi Haematologica 55 681–697 (1970)*

9 LIOTTI F S, GIOVANNINI E e MIGLIORINI BRUSCHELLI G Su talune proprietà dei fattori eritrostimolanti presenti nel plasma, nel rene e nella milza del coniglio *Riv Biol 65 3–51 (1972)*

10 LOWY, P H and BORSOOK, H Preparation and properties of erythropoietin concentrates from rabbit plasma and human urine, in JACOBSON and DOYLE Erythropoiesis, *33–42 (Grune & Stratton New York 1962)*

11 LOWY, P H and KEIGHLEY, G Inactivation of erythropoietin by Koshland's tryptophan reagent and by membrane filtration Biochim biophys Acta 160 413–419 (1968)

12 WONG, K K, ZANJANI E D, COOPER G W, and GORDON, A S The renal erythropoietic factor V Studies on its purification Proc Soc exp Biol Med *128* 67–70 (1968)

13 ZANJANI E D, CONTRERA, J F, GORDON A S, COOPER, G W, WONG, K K, and KATZ, R The renal erythropoietic factor (REF) III Enzymatic role in erythropoietin production Proc Soc exp Biol Med *125* 505–508 (1967)

Dr ELVIO GIOVANNINI, Istituto di Biologia generale dell Università Via del Giochetto *I-06100 Perugia* (Italia)

Acta haemat 55 109-117 (1976)

Cephalothin-Induced Immune Hemolytic Anemia

M. Jeannet, A. Bloch, J. M. Dayer, J. J. Farquet, J. P. Girard and A. Cruchaud

Department of Medicine, Hôpital cantonal, Geneva

Key words Cephalothin · Drug induced hemolytic anemia · Hemolytic anemia · Immune reactions

Abstract A patient with renal disease developed Coombs positive hemolytic anemia while receiving cephalothin therapy. An anti-cephalothin IgG antibody was detected in the patient's serum and in the eluates from her erythrocytes. In addition, nonimmunologic binding of normal and patient's serum proteins to her own and cephalothin-coated normal red cells was demonstrated. Skin tests and *in vitro* lymphocyte stimulation revealed that the patient was sensitized to cephalothin and also to ampicillin. Careful investigation of drug induced hemolytic anemias reveals the complexity of the immune mechanisms involved

Drug-induced immune hemolytic anemias are uncommun despite the fact that many drugs lead to the development of antibodies. However, some of these drugs, such as penicillin, bind firmly to red cells *in vitro* and more than 20 cases of penicillin-induced hemolytic anemia have been reported [11]. Cephalothin and other cephalosporins which are chemically related to penicillin, also bind to red cells *in vitro*. Cephalothin sodium (Keflin) has been associated with a positive direct Coombs test in many patients receiving this antibiotic [4, 7] but only 2 cases of cephalothin and 1 case of cephalexin-induced immune hemolytic anemia have been reported as yet [2, 5]. The purpose of this paper is to report an additional case of Coombs-positive hemolytic anemia related to cephalothin therapy and to define the characteristics of immunohematologic reactions to this drug

Table II Coombs test after incubation of erythrocytes with cephalothin or penicillin using patient's serum, patient's erythrocyte eluate or normal serum

	Cephalothin-coated erythrocytes			Penicillin-coated erythrocytes			Normal erythrocytes		
	broad-spectrum	*IgG*	*C3*	*broad-spectrum*	*IgG*	*C3*	*broad spectrum*	*IgG*	*C3*
Patient's serum	1 512	1 512	1 32	1 2	ND	ND	–	–	–
Patient's eluate	1 4	ND	ND	–	ND	ND	–	ND	ND
Normal serum 1	1 256	1 256	1 32	1 4	ND	ND	–	ND	ND
Normal serum 2	1 128	1 256	1 32	–	ND	ND	–	ND	ND

ND = Not done

patient's erythrocytes agglutinated cephalothin-coated erythrocytes, but did not agglutinate penicillin-coated erythrocytes or normal erythrocytes. That the antibody in patient's serum and eluate belonged to the IgG class was confirmed by the absence of modification of the agglutination titer of cephalothin-coated erythrocytes following 2-mercaptoethanol treatment. Two normal sera from blood donors were used as controls. Both were found to agglutinate cephalothin-coated erythrocytes almost as strongly as the patient's serum using the same broad-spectrum, anti-IgG and anti-C3 antisera (table II).

Fractionation studies. 10/ml of the patient's serum obtained 15 days following interruption of therapy and when direct Coombs test was strongly positive were chromatographed on Sephadex G-200. The elution diagram was arbitrarily divided into 5 fractions which were concentrated to obtain twice the original volume of serum. These fractions were assayed with cephalotin-coated erythrocytes and antisera to IgG, IgA, IgM, C3, *transferrin and β-lipoprotein*. Fractions III and IV which disclosed strong agglutination with anti-IgG, anti-IgA and anti-transferrin were pooled and recycled on Sephadex G-200. Three fractions were obtained which were analyzed by immunoelectrophoresis and by radial diffusion in agar (Tri-Partigen, Behringwerke, Marburg/Lahn, FRG) to evaluate their content in immunoglobulins, C3 and transferrin. These fractions were again assayed with cephalothin-coated erythrocytes and antisera. The same procedure was performed with a pool of two normal blood donors

III Serum proteins present in fractions from patient's and normal sera following fractio Sephadex G 200

	Radial immunodiffusion					Immunoelectrophoresis				
	total	IgG	IgA	IgM mg ml	trans-ferrin	IgG	IgA	IgM	trans-ferrin	β-lipo prote
tions from patient's serum										
	ND	4 0	0 34	0 08	0	+	(+)	−	−	−
	ND	17 0	2 68	0	0	+ + +	+ + +	−	−	−
	ND	27 7	0 66	0	0	+ + + +	+	−	−	−
tions from a pool of two normal sera										
	3 0					−	−	−	−	−
	27 0		ND			+ +	+	−	+	−
	18 0					+ +	+ +	−	−	−

le II Coombs tests after incubation of cephalothin-coated erythrocytes[1] using fractions fro and normal sera

sera[2]	Broad spectrum	IgG	IgM	IgA	Trans-ferrin	β-lipo-protein
ctions from patient's serum	+ + +	+ + +	−	+ + +	+ + +	+ +
	+	+	−	+ + +	+ +	+
	+	+	−	+ + +	+	+
	+	+ + +	−	+ +	−	−
ctions from normal sera	+ + +	+ + +	−	+ + +	+ + +	+ +
	−	+	−	+	−	−
	+	+	−	+	−	−
	+	+ +	−	−	−	−

[1] Non-coated erythrocytes were negative with all antisera
[2] All antisera used ■ dilution 1 8

Table III shows the concentrations of the different serum proteins found in the three fractions used IgG was recovered mainly in fractions II and III IgA was found in all fractions but especially in fraction II When incubated with the three fractions, cephalothin-coated erythrocytes were found to absorb serum proteins from each fraction in a nonspecific

Table V. Patient's lymphocyte stimulation in culture using penicillin, ampicillin and cephalothin as antigen performed on 2 different days (mean of 2 tests)

	16.8.70 cpm	18.9.70 cpm
Lymphocytes alone	513	472
Lymphocytes + penicillin G	971	725
Lymphocytes + ampicillin	1,119	884
Lymphocytes + cephalothin	1,096	1,216
Lymphocytes + PPD (10 μ)	1,582	1,390
Lymphocytes + phytohemagglutinin	63,620	54 620

way. Moreover (table IV), no clear-cut difference could be seen between the fractions from the patient's serum and those from normal serum. The serum proteins most readily absorbed by this apparently nonimmunologic mechanism were IgG, IgA, transferrin and β-lipoprotein.

Evidence for sensitization to cephalothin. Skin tests were performed in the patient, 9 days after therapy with cephalothin had been interrupted. A positive skin test was observed after intradermal injection of penicilloyl-polylysine, 250 mmol/ml), the last test was stronger than the others. Lymphocyte culture tests were also performed on two different days, using the same three drugs as antigens (table V). All three tests induced moderately positive lymphocyte stimulation.

Discussion

The reported incidence of positive Coombs test in patients receiving cephalothin has varied between 3.4 and 75% [4, 7, 9, 11]. These reactions were often weak and occurred from one to a few days after starting treatment. They were not associated with hemolysis. In some studies, the dosage and duration of therapy were found to influence the incidence of positive reactions. A large percentage of patients with positive Coombs tests had renal disease with high blood levels of cephalothin secondary to reduced renal excretion. As suggested by GRALNICK *et al.* [4], hypoalbuminemia may also favor the occurrence of positive Coombs tests, the albumin fraction being responsible for over 95% of the serum binding of cephalothin [6].

Previously proposed mechanisms regarding the pathogenesis of posi-

tive Coombs test without hemolysis, occurring in patients receiving cephalothin include (1) a nonimmunologic binding of serum proteins to red cells [4, 7, 11] and (2) an immune reaction involving an antibody cross-reacting with cephalothin- or penicillin-coated red cells [4, 8, 11] As indicated by SPATH *et al* [11], both mechanisms seem to occur in parallel in these patients Nonimmunological absorption of proteins, especially albumin, to cephalothin-coated red cells could be demonstrated *in vitro*, multiple absorptions of a serum with cephalothin-sensitized erythrocytes revealed that these cells absorb IgG and other proteins, including complement (C3) and albumin [11] Our own data confirm this nonimmunologic binding of proteins and revealed that IgA, transferrin and β lipoprotein may also be absorbed from normal sera by cephalothin-coated erythrocytes (table III) In addition SPATH *et al* [11] have demonstrated that an immune mechanism may also cause positive direct antiglobulin tests *in vivo* during cephalothin therapy Eluates from the patient s erythrocytes react with cephalotin and penicillin sensitized erythrocytes This was not a nonspecific absorption of protein because eluates from red cells sensitized with an anti Rh IgG isoantibody would not bind IgG to cephalothin-coated red cells lacking the appropriate antigen In addition, SPATH *et al* [11] showed that the sera of 4 patients having a positive antiglobulin test all contained high titers of IgG cephalothin antibodies, whereas in the other treated patients IgM antibodies were found in 88% and IgG antibodies in only 25% of cases

Despite the frequent occurrence of a positive direct Coombs test in patients receiving cephalothin only 2 cases of hemolytic anemia associated with cephalothin have been described so far [5] As in our case, erythrocytes from these patients were strongly positive in the direct Coombs test with broad spectrum anti human globulin reagents and with a specific anti IgG antiserum Our patient s erythrocytes were also strongly agglutinated by an anti-complement (C3) reagent Weak reactions were also obtained with anti IgM, anti IgA and anti transferrin reagents, suggesting the simultaneous occurrence of nonimmunological binding of serum proteins In our case as in the others, a specific IgG cephalothin antibody was demonstrated in eluates from patients' erythrocytes However, as mentioned above this specific immune reaction was also observed in patients with positive direct Coombs test without hemolysis and therefore cannot be used as a definitive proof that cephalothin caused the hemolytic anemia Presumably, as suggested by WORLLEDGE [12] the quality and quantity of the antibody are also important.

ANNOUNCEMENT

Indian Council of Medical Research

Colonel Amir Chand Trust Prizes for Medical Research

t.-Colonel Amir Chand, lately Principal of Lady Hardinge Medical ge, New Delhi, has donated Rs. 50,000/- to the Indian Council of al Research for creating a prize fund. From the interest earned by n prizes are to be awarded for the best published research work in medical rs. The Governing Body of the Council has constituted a Trust called Colonel Amir Chand Trust for the administration and management of the t.

The prizes are awarded annually on an All India basis for the best published rch work in any subject in the field of medical sciences including clinical arch. The term Clinical Research covers research into the mechanism causation of disease and its prevention and cure, and includes work on nt in hospitals, field studies in epidemiology and social medicine and rvations in general practice.

It has been decided to award *four prizes* in 1959 each of Rs. 300/- to s of not more than ten years' standing, for the best research papers in ... zes will be known ... GRADUATES ... Selection Board.

In the case of joint authorship of a publication the prize shall be divided een the authors in such proportion as the Selection Board may decide. r ... of the person who applies for the prize should be clearly indicated to make it easy to determine whether the major part of the work has d ne by that person.

THE AWARD of the prizes will be announced at the annual meetings of Council's Scientific Advisory Board and Advisory Committees in /December 1959.

CANDIDATES for award of prizes are required to submit 10 REPRINTS of r papers published during 1958. These should be sent to the DIRECTOR, IAN COUNCIL OF MEDICAL RESEARCH, P. O. BOX 494, NEW DELHI, so as to reach NOT LATER THAN 1ST AUGUST 1959. THE PAPERS should be accompanied a short biographical sketch and two copies of passport size photographs of candidate/candidates concerned.

NEWS & NOTES

In the April [illegible] issue of our journal on page 148 the following news item was published under the heading "Treatment of leprosy patients under the Ayurvedic System". In this connection Dr Wardekar, the Secretary of the Gandhi Memorial Leprosy Foundation, Wardha, has very kindly sent us the following information:—

The Gandhi Memorial Leprosy Foundation (Leprosy section of the Gandhi Smarak Nidhi) does not have any proposal to start a [illegible] bedded hospital for routine Ayurvedic Treatment. It has decided only to undertake for a period of 2 years a trial of Ayurvedic drugs with the sole object of studying the efficacy of these drugs on a few selected cases of leprosy. Arrangements have been made to accommodate only 10 male and 10 female patients. The trial will be conducted by a Registered body to be formed by Shri Parthasarathy Pandit for this purpose.

open A separate consideration is perhaps indicated for the combined deficiency of factors VII and X together with carotid body tumors described in several members of a large kindred [20] The cases of hemophilia B with associated factor VII deficiency are also doubtful since they could represent cases of hemophilia B_M [15, 28]

Combined deficiency of factors VII and VIII has been satisfactorily documented only in 1 patient [5] and suspected in 3 additional cases [2, 19] In only 1 instance the defect has been demonstrated in family members of the propositus [19] Furthermore, even in the case shown to have low factors VIII and VII by specific assay [5], the possibility of a non hemophilic factor VIII defect was not ruled out [5]

The object of the present study is to report a patient who presented a mild hemophilia A and moderate factor VII deficiency The same defect was present in several family members of both sexes Therefore, this seems to be the first family presenting a combined defect of factors VII and VIII

Case Report

The propositus is a 22 year old male who was first seen by us in Padua on December 17, 1974 and had been followed by one of us (R V) for the past 4 years The family history was partially positive for bleeding tendency (fig 1) The mother presented easy bruising menomethrorrhagia and *post partum* bleeding A maternal aunt presented menomethrorrhagia A maternal cousin had bled excessively after brain surgery for a benign tumor and easy bruising Another maternal cousin was known to be prone to gingival bleeding The parents were not consanguineous and came from different areas

At the age of 6 the patient bled profusely after tonsillectomy Bleeding stopped after two local packings and a fresh blood transfusion The blood transfusion, however, had to be interrupted because of a reaction At the age of 7 the patient was involved in a car accident and sustained a fracture of the left femur and a laceration of the upper lip Bleeding from the wound was marked He was admitted to a hospital in Turin where he was sutured and a cast was applied The healing of the fracture was very slow since it required a 3 month period before the cast could be removed At the age of 8 the patient had an abdominal trauma which caused melena Bleeding subsided after a few days of bed rest At the age of 10 the patient presented excessive bleeding after a deciduous tooth extraction At that time he was suspected to have hemophilia A

At the age of 18 the patient presented severe bleeding after a molar tooth extraction He had to be admitted to a local hospital and bleeding subsided only after transfusion of fresh frozen plasma During this admission, a routine coagulation study revealed a moderate factor VII defect together with a mild prolongation of the partial thromboplastin time This latter finding remained unexplained

Easy bruising and epistaxes have been present since childhood Hematuria and hemarthrosis have never occurred At the time of study there were no bleeding manifestations

Physical examination was negative Routine laboratory tests including a battery of liver function test were all within normal limits

Material and Methods

Material and methods have been described in detail elsewhere [6-8] Only new data will be given herein

Factor VIII associated antigen was evaluated according to a previously reported modification of the method proposed by LAURELL [14] Anti factor VII activity was evaluated by incubating in plastic tubes at 37°C for 1 h, a mixture of patient s plasma and fresh normal plasma and a mixture of classical factor VII deficiency plasma and fresh normal plasma. Factor VII was then assayed at 60-min intervals in both mixtures The factor VII deficient plasma used in these experiments belonged to 2 patients with classical factor VII deficiency previously reported by us The factor VII level in such plasms is about 3% of normal [10] Anti factor VIII inhibitor activity was evaluated according to a modification of the method proposed by BIGGS and BIDWELL [1] A lyophilized factor VIII concentrate preparation (Kryobulin 250, Immuno Wien) was reconstituted according to the manufacturer s instructions with 30 ml of distilled water 1 ml of this preparation was then diluted 1 3 with normal saline 0 1 ml of such diluted preparation was then added to 0 9 ml of patient plasma or 'fake' plasma The fake plasma was obtained by mixing 0 2 ml of normal plasma and 0 8 ml of severe hemophilia A plasma. The factor VIII level of such mixed fake plasma was 20% of normal After a 60-min incubation period at 37°C, factor VIII was assayed in a one-stage system in both mixtures

The neutralization test was carried out according to a modification of the method proposed by LEVANON *et al* [21] The test was carried out as follows *Stage 1* anti factor VII antiserum[a] (0 05 ml) was added to 0 9 ml of the test plasma This dilution inactivated after 1 h approximately 90-95% of factor VII in normal plasma The mixture was incubated for 1 h at 37°C and then centrifuged at 5 000 rpm for 20 min. *Stage 2* 0 25 ml of the supernatant was then transferred to a second tube containing 0 25 ml of pooled normal plasma This mixture was also incubated and centrifuged in the same way as in Stage 1 *Stage 3* the supernatant was assayed for residual factor VII activity A calibration curve was constructed by diluting pooled normal plasma to have factor VII concentration over the range of 100-5%

Ristocetin platelet aggregation was carried out as previously reported [16] The final concentration of ristocetin in the cuvette was 1 5 mg/ml.

Results

The coagulation study in our propositus is summarized in table I Glass clotting time was at the upper limits of normalcy Prothrombin time was slightly prolonged too and remained so even using a factor-V- and fibrinogen-

[a] The anti-factor VII antiserum used in these experiments was kindly supplied by Drs BECKER and HEIMBURGER of Behringwerke Research Laboratories, Marburg (FRG)

open A separate consideration is perhaps indicated for the combined deficiency of factors VII and X together with carotid body tumors described in several members of a large kindred [20] The cases of hemophilia B with associated factor VII deficiency are also doubtful since they could represent cases of hemophilia B_M [15, 28]

Combined deficiency of factors VII and VIII has been satisfactorily documented only in 1 patient [5] and suspected in 3 additional cases [2, 19] In only 1 instance the defect has been demonstrated in family members of the propositus [19] Furthermore, even in the case shown to have low factors VIII and VII by specific assay [5], the possibility of a non-hemophilic factor VIII defect was not ruled out [5]

The object of the present study is to report a patient who presented a mild hemophilia A and moderate factor VII deficiency The same defect was present in several family members of both sexes Therefore, this seems to be the first family presenting a combined defect of factors VII and VIII

Case Report

The propositus is a 22 year old male who was first seen by us in Padua on December 17, 1974 and had been followed by one of us (R V) for the past 4 years The family history was partially positive for bleeding tendency (fig 1) The mother presented easy bruising, menomethrorrhagia and *post partum* bleeding A maternal aunt presented menomethrorrhagia A maternal cousin had bled excessively after brain surgery for a benign tumor and easy bruising Another maternal cousin was known to be prone to gingival bleeding The parents were not consanguineous and came from different areas

At the age of 6 the patient bled profusely after tonsillectomy Bleeding stopped after two local packings and a fresh blood transfusion The blood transfusion, however, had to be interrupted because of a reaction At the age of 7 the patient was involved in a car accident and sustained a fracture of the left femur and a laceration of the upper lip Bleeding from the wound was marked He was admitted to a hospital in Turin where he was sutured and a cast was applied The healing of the fracture was very slow since it required a 3 month period before the cast could be removed At the age of 8 the patient had an abdominal trauma which caused melena Bleeding subsided after a few days of bed rest At the age of 10 the patient presented excessive bleeding after a deciduous tooth extraction At that time he was suspected to have hemophilia A

At the age of 18 the patient presented severe bleeding after a molar tooth extraction He had to be admitted to a local hospital and bleeding subsided only after transfusion of fresh frozen plasma During this admission, a routine coagulation study revealed a moderate factor VII defect together with a mild prolongation of the partial thromboplastin time This latter finding remained unexplained

Easy bruising and epistaxes have been present since childhood Hematuria and hemarthrosis have never occurred At the time of study there were no bleeding manifestations

Physical examination was negative. Routine laboratory tests including a battery of liver function test were all within normal limits.

Material and Methods

Material and methods have been described in detail elsewhere [6–8]. Only new data will be given herein.

Factor VIII-associated antigen was evaluated according to a previously reported modification of the method proposed by LAURELL [14]. Anti-factor VII activity was evaluated by incubating in plastic tubes at 37°C for 1 h, a mixture of patient's plasma and fresh normal plasma and a mixture of classical factor VII deficiency plasma and fresh normal plasma. Factor VII was then assayed at 60-min intervals in both mixtures. The factor VII-deficient plasma used in these experiments belonged to 2 patients with classical factor VII deficiency previously reported by us. The factor VII level in such plasms is about 3% of normal [10]. Anti factor VIII inhibitor activity was evaluated according to a modification of the method proposed by BIGGS and BIDWELL [1]. A lyophilized factor VIII concentrate preparation (Kryobulin 250, Immuno, Wien) was reconstituted according to the manufacturer's instructions with 30 ml of distilled water. 1 ml of this preparation was then diluted 1:3 with normal saline. 0.1 ml of such diluted preparation was then added to 0.9 ml of patient plasma or 'fake' plasma. The fake plasma was obtained by mixing 0.2 ml of normal plasma and 0.8 ml of severe hemophilia A plasma. The factor VIII level of such mixed fake plasma was 20% of normal. After a 60-min incubation period at 37°C, factor VIII was assayed in a one-stage system in both mixtures.

The neutralization test was carried out according to a modification of the method proposed by LEVANON *et al.* [21]. The test was carried out as follows: *Stage 1*: anti factor VII antiserum[a] (0.05 ml) was added to 0.9 ml of the test plasma. This dilution inactivated after 1 h approximately 90–95% of factor VII in normal plasma. The mixture was incubated for 1 h at 37°C and then centrifuged at 5 000 rpm for 20 min. *Stage 2*: 0.25 ml of the supernatant was then transferred to a second tube containing 0.25 ml of pooled normal plasma. This mixture was also incubated and centrifuged in the same way as in Stage 1. *Stage 3*: the supernatant was assayed for residual factor VII activity. A calibration curve was constructed by diluting pooled normal plasma to have factor VII concentration over the range of 100–5%.

Ristocetin platelet aggregation was carried out as previously reported [16]. The final concentration of ristocetin in the cuvette was 1.5 mg/ml.

Results

The coagulation study in our propositus is summarized in table I. Glass clotting time was at the upper limits of normalcy. Prothrombin time was slightly prolonged too and remained so even using a factor-V- and fibrinogen-

[a] The anti-factor VII antiserum used in these experiments was kindly supplied by Drs. BECKER and HEIMBURGER of Behringwerke Research Laboratories, Marburg (FRG).

Table I Coagulation study in the propositus

Test	Propositus	Normal value
Clotting time, min	10	5–10
Bleeding time, min	4	2–5
Platelet count	175 000	150 000–350,000
Clot retraction	complete in 8 h	complete in less than 12 h
Platelet adhesiveness, %	35	20–50
Platelet aggregation to ristocetin (maximal amplitude of the curve), %	65	70
Partial thromboplastin time, sec	52 5	32–42
Prothrombin consumption, %	90	>90
Thromboplastin generation test	60 sec in 10 min	<20 sec in 6–8 min
Prothrombin time, sec	17 2	14
Stypven-cephalin clotting time, sec	12	12
Thrombotest sec	53	43
Normotest, sec	36	28
PP test, sec	37 1	29 4
Factors, II, V, IX, X, XI, XII, %	normal	60–160
Factor VII, %	35[1]	85–125
Factor VIII, %	18[1]	60–160
Fibrinogen, mg%	400	250–500
Thrombin time, sec	19	18–25
Euglobulin lysis time, h	20	10–30
Thromboelastogram r	21	10–20
K	12	6–12
ma	52	50–66

[1] Average of several assays

containing thromboplastin Recalcification and partial thromboplastin times were moderately prolonged The partial thromboplastin time was corrected by the addition of adsorbed normal plasma but not by the addition of normal serum or hemophilia A plasma (table II) The thromboelastogram showed a slightly prolonged 'r + K', whereas 'ma' was normal

On the contrary, Stypven-cephalin clotting time was normal Prothrombin time derivative tests (PP test, Thrombotest and Normotest) were all slightly prolonged too TG test was defective and was corrected by the substitution of patient adsorbed plasma with adsorbed normal plasma (fig 2) Prothrombin consumption was borderline (90%)

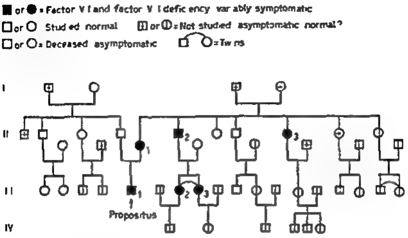


Fig 1 Fam ly ped gree Bes des the propositus 5 other fam ly members were affected The pattern of hered tary transm ss on is autosomal dom nant All persons studied on the paternal side were normal.

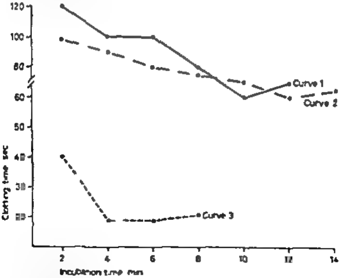


Fig 2 Thromboplastin generation test in the propos tus Curve 1 refers to the basal curve Curve II was obta ned after the subst tution of pat ent s serum w th normal serum Curve 3 was obta ned after the subst tut on of pat ent s adsorbed plasma with adsorbed normal plasma

Table II Partial thromboplastin time (PTT) cross-correction studies in the propositus

Mixtures (equal parts)	PTT of mixture, sec	PTT of reference plasma, sec
Propositus plasma	52.5	–
Propositus plasma + adsorbed normal plasma	37.5	–
Propositus plasma + normal serum	70.1	–
Propositus plasma + hemophilia A plasma	76	106

Table III Factor VII and VIII inhibitors Lack of anti factor VII or anti factor VIII inhibitor in the propositus' plasma

	Incubation time, min		
	0	60	120
Factor VII			
Level of a 1 2 mixture of patient s plasma + *fresh normal plasma*	80	86	84
Level of a 1 2 mixture of severly *factor-VII deficient plasma + fresh normal plasma*	60	58	66
Factor VIII			
Concentrate + propositus plasma		70	
Concentrate + fake plasma		78	

Platelet and vascular tests were all within normal limits Fibrinogen was normal and there was no hyperfibrinolysis Factor VII was moderately reduced (35% of normal) Factor VIII was moderately decreased too (18% of normal) Factors II, V, IX, X, XI and XII were all within normal limits Factor-VIII-associated antigen was at the lower limits of normalcy Factor VII protein as assayed by neutralization test was definitely higher than the factor-VII-clotting counterpart (fig 3)

No anti-factor VII or anti-factor VIII inhibitors were found in the patient's plasma (table III)

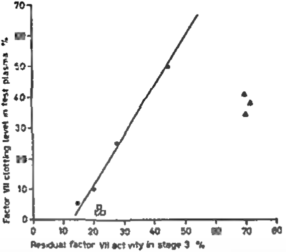


Fig. 3. Relationship of factor VII-clotting activity and residual factor VII activity in antibody neutralization assay (stage 3) in the propositus, his mother, another affected member (▲) and in 3 patients with severe factor VII deficiency (□). The residual factor VII activity (stage 3) observed in the propositus and in the other two affected members is considerably higher than expected. The level observed is similar to that observed in normal subjects. This indicates that a normal or nearly normal factor VII antigen is present in the propositus' plasma. The dots and the best fitted line represent the calibration curve obtained by serial dilutions of pooled normal plasma.

Table IV. Factor VII activity, factor VIII activity and factor VIII antigen in the affected family members (fig. 1) of the propositus

Patient	Simplastin % of normal	Thrombo-plastin % of normal	Factor VIII activity	Factor VIII antigen
Propositus (III$_1$)	40	11	11	60
Father	100	100	105	160
Mother (II$_1$)	54	47	25	110
II$_2$	54	45	32	130
II$_3$	65	64	26	100
III$_2$	46	38	22	100
III$_3$	52	47	20	110
Normal	85–120	85–120	60–160	60–160

Factor VII was assayed using a rabbit brain + lung thromboplastin (Simplastin) and a human brain thromboplastin (Manchester Thromboplastin). The results are the average of at least two determinations carried out on different occasions.

The daily intravenous administration of vitamin K (10 mg) for a few days failed to correct the factor VII defect

Mildly prolonged partial thromboplastin and prothrombin times due to reduced factor VII and VIII activity were found also in 5 family members including the mother Factor-VIII-associated antigen was normal in all affected patients (table IV) Factor VII antigen was assayed by means of the neutralization test in 2 patients, besides the propositus, and was normal or near normal

The patients affected turned out to be those who had presented some bleeding manifestations Only 1 patient (case II_3, fig 1) was affected and had so far no bleeding symptoms This patient, however, has never undergone surgery or major trauma during his life and therefore his hemostatic system has probably not been challenged so far

Discussion

The laboratory findings are consistent with the presence of a first- and second stage defect Specific assays demonstrated that the defect was due to the concomitant lack of factors VII and VIII The possibility our patient had von Willebrand s disease, together with a factor VII deficiency, may be safely ruled out because of the normal bleeding time, normal pletelet adhesiveness, normal factor VIII antigen and normal platelet aggregation to ristocetin The presence of normal or low-normal factor-VIII-associated antigen in the plasma of our propositus seems to suggest that the defect is of the hemophilia type This is the first demonstration of a normal factor-VIII-associated antigen in combined factor VII and factor VIII deficiency The factor VIII antigen level found in our propositus is slightly lower as compared to that found in his mother and other maternal relatives The level appears to be at the lower limits of normalcy and has remained so in repeated assays We do not know if this has any significance

It is to be remembered in this regard that a normal factor-VIII-associated antigen has been demonstrated already in all 3 patients with combined deficiency of factor V and factor VIII so far examined [*9, 17, 29*]

Our impression is that, at least as far as our family is concerned, the association between factors VII and VIII is not casual The statistical propability for such an association is indeed very low Factor VII deficiency is a rare disorder Only about 100 propable or sure cases have been described so far in the world literature and only 5 sure homozygotes have been describ-

ed in Italy together with only about 20 heterozygotes (10, 11, 22) Hemophilia A is a less rare condition and seems to be present in Italy in 1 out of 20 000 people Given a population of 55 million, approximately 3,000 hemophiliacs appear to be living at the present time in Italy It would be extremely rare for a female patient to have a factor VII defect and at the same time to be a carrier of a factor VIII defect or for a male patient to have hemophilia A and a factor VII defect Furthermore, against the causal association hypothesis remains the fact that no isolated factor VII or factor VIII deficiency was found in the family members of the maternal side of our propositus Finally, the presence of a factor VIII defect in females is not either consistent with the association of the two defects

The most likely explanation is to admit that our patients 'lack' a gene or a system of genes on an autosomal chromosome involved in the activation of both factors VII and VIII If this is so, we may suspect that they have a normal factor VII antigen too In agreement with these assumptions are the neutralization test studies The residual factor VII activity in stage 3, was defenitely higher than expected, indicating the presence of a normal or nearly normal factor VII antigen in stage-1 samples In this regard, it is interesting to note that slightly higher levels of factor VII antigen as compared to factor VII activity were also present in our 3 patients with severe factor VII deficiency This is in agreement with the findings of others [3, 18, 21] Our propositus seems to have a structural abnormality of factors VII and VIII whereby a normal or near normal synthesis of the two proteins has occurred but an incomplete activation of same has ensued

The hereditary pattern in our family seems autosomal dominant No other explanation would suit the findings Therefore we would have to admit that a non von-Willebrand factor VIII defect, under certain circumstances, may be transmitted as an autosomal dominant trait

On the basis of the data presented herein it may be inferred that the family presented by CONSTANDOULAKIS [2] met fully these assumptions too In this case the mother of the propositus had a low factor VII and a prolonged glass clotting time In this pedigree 2 patients with apparent isolated mild factor VII deficiency are reported too However, factor VIII was not assayed in these latter patients and therefore a mild deficiency could have been missed On the contrary, the patient presented by GASTON *et al* [5] could represent a second type of combined factor VII and factor VIII deficiency, the casual association type In this family, in fact, the only patient affected appears to be a hemophiliac who has probably inherited one defective gene controlling factor VII from either of his parents So he appears to be a

hemophiliac and at the time a heterozygote for factor VII deficiency Unfortunately neither the father nor the mother were studied and so no data on the hereditary transmission is obtainable from this pedigree

A similar classification into two different types of defect has been recently proposed for combined factor V and factor VIII deficiency [9, 12] This latter defect, however, is transmitted as an autosomal recessive trait The data presented in this paper clearly indicate the existence of another factor VIII defect and of another factor VIII inheritance pattern

References

1 BIGGS R and BIDWELL, E A method for the study of antihemophilic globulin inhibitors with reference to six cases Br J Haemat 5 379 (1959)
2 CONSTANDOULAKIS, M Familial hemophilia and factor VII deficiency J clin Path 11 412 (1958)
3 DENSON, K W, CONRAD, J and SAMAMA, M Genetic variants of factor VII Lancet I 1234 (1972)
4 FISCHER, M und ZWEYMÜLLER, E Kongenitaler kombinierter Mangel der Faktoren II, VII und X Z Kinderheilk 95 309 (1966)
5 GASTON, L W, MACH, B F, and BECK, W S Hemophilia A and concurrent factor VII deficiency New Engl J Med 274 1078 (1961)
6 GIROLAMI, A, STICCHI, A., LAZZARIN, M, and SCARPA, R Congenital hypoprothrombinemia Case report Acta haemat 44 164 (1970)
7 GIROLAMI, A, MOLARO, G, LAZZARIN, M, SCARPA, R, and BRUNETTI, A A new haemorrhagic condition due to the presence of an abnormal factor X (factor X Friuli) A study of a large kindred Br J Haemat 19 179 (1971)
8 GIROLAMI, A, VENTURELLI R, RIGHINI, F, and SCARPA R Parahaemophilia A case report Acta haemat 46 242 (1971)
9 GIROLAMI A, BRUNETTI, A e PATRASSI, G M La deficienza congenita combinata del fattore V e del fattore VIII Paraemofilia ed emofilia A Biologica lat 23 149 (1972)
10 GIROLAMI, A, CATTOROZZI, G, MENOARDA, G, and LAZZARIN, M Congenital factor VII deficiency A case report Blut 27 236 (1973)
11 ... SANTINI, G Congenital ... s belonging to two
12 GIROLAMI, A, BRUNETTI, A, and DE MARCO, L Congenital combined deficiency of factor V and factor VIII in a male born from a brother sister incest Blut 28 33 (1974)
13 GIROLAMI, A and BAREGGI, G Nor... genital deficiency of factor V and fac...
14 GIROLAMI A, STICCHI, A, BARBUI, ... assay An evaluation of several methods using whole plasma Blut 29 309 (1974)

15 Girolami, A , Cella, G and Patrassi, G M L'emofilia B_M (coagulopatia da fattore IX anomalo) Haematologica 59 91 (1974)
16 Girolami, A., De Marco, L., Virgolini, L., Peruffo R., and Fabris, F Platelet adhesiveness and aggregation in congenital afibrinogenemia. Blut 30 [illegible] (1975)
17 Girolami, A , Gastaldi, G , Patrassi, G [illegible], and Galletti A Combined congenital deficiency of factor V and factor VIII Report of a further case with some considerations on the hereditary transmission of this disorder Acta haemat 55 (in press 1976)
18 Goodnight, S H , Feinstein, D I , Osterud, B , and Rapaport, S I Factor VII antibody neutralization material in hereditary and acquired factor VII deficiency Blood 38 1 (1971)
19 Koller, F Physiology and pathology of blood coagulation review of literature of 1957 Thromb Diath. haemorrh. 2 358 (1958)
20 Kroll, A J , Alexander, B , Cochios, F , and Pecht, L Hereditary deficiencies of clotting factors VII and X associated with carotid body tumors. New Engl J Med. 270 6 (1964)
21 Levanon M , Rimon, S , Shaki, M , Ramot, S , and Goldberg, E. Active and inactive factor VII in Dubin Johnson syndrome with factor VII deficiency and on coumadin administration Br J Haemat. 23 669 (1972)
22 Mandelli, F , Mariani, G , Gandolfo, G M et Isacchi, G Considérations sur deux cas d'hypoconvertinémie congénitale Nouv Revue fr Hémat 9 485 (1969)
23 Mc Millan C. W and Roberts, H R Congenital combined deficiency of coagulation factors II, VII IX and X New Engl. J Med 274 1313 (1966)
24 Menaché, D et Makin, S Un nouveau cas de déficit associé aux facteurs VIII et V Path. Biol , Paris 16 969 (1968)
25 Saito, H , Shioya H , Koia, K., Kamika, T., and Katsumi, O Congenital combined deficiency of factor V and factor VIII A case report and the effect of transfusion of normal plasma and hemophilic blood Thromb Diath. haemorrh. 22 316 (1969)
26 Seligsohn, U and Ramot, B Combined factor V and factor VIII deficiency Report of four cases. Br J Haemat 16 475 (1969)
27 Smit Sibinga, C. T , Gökemeyer, J D M , Tenkate, L. P., and Bos-von Zwol, F Combined deficiency of factor V and factor VIII Report of a family and genetic analysis. Br J Haemat. 23 467 (1972)
28 Verstraete, M , Vermylen, C., and Vandenbroucke, J Hemophilia B associated with a decreased factor VII activity Am. J med. Sci 74 243 (1962)
29 Zimmerman, T S , Ratnoff, O D., and Powell, A E. Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and an acquired circulating anticoagulant against antihemophilic factor J clin. Invest. 50 244 (1971).

Prof A Girolami Istituto di Semeiotica Medica, Via Ospedale Civile 43, Padova (Italia)

Book Review · Buchbesprechung · Livre nouveau

J M UNSELD **Blutersatz durch stromafreie Hämoglobinlösung** Ergebnisse tierexperimenteller Untersuchungen Springer, Berlin 1974 VII + 90 pp, 17 fig, DM 32 -/US$ 13 10

Der Autor legt die vollstandige Dokumentation über seine Untersuchungen mit einer stromafreien Hamoglobinlosung am normovolamischen und hypovolamischen Zwergschwein vor Zum Vergleich diente eine Albuminlösung bzw bei den durch Blutung in den hamorrhagischen Schock versetzten Tieren die Retransfusion des Blutes Es wurde das Verhalten verschiedener Kreislaufgrossen, des O_2-Transport vermogens, der O_2 Aufnahme und der Nierenfunktion untersucht In Bestatigung früherer Befunde von RABINER erwies sich die Hamoglobinlosung nicht als nephrotoxisch Im hamorrhagischen Schock hatte sie kurzfristig eine dem Vollblut ahnliche, den Plasmaexpandern in bezug auf O_2 Transport überlegene Wirkung Durch die – speziesabhangige – kurze Halbwertszeit fur das Verschwinden aus dem Plasma (beim Schwein ca $3^1/_2$ h) ist die Anwendbarkeit allerdings beschrankt Die Befunde werden eingehend diskutiert, wobei jedoch die neueste Literatur keine Berucksichtigung findet, da die Arbeit offenbar schon 1972 abgeschlossen worden ist Aus der Nichtberucksichtigung wichtiger alterer Arbeiten – z B derjenigen von BUNN *et al* – erklart sich wahrscheinlich die etwas einseitige Betrachtungsweise So halten die Angaben uber die Elimination der Hamoglobinlösung nicht jeder Kritik stand Ähnliches gilt für die Bemerkungen uber die Sauerstoffabgabe Das kleine Buch dem eine kurze englische Zusammenfassung beigegeben ist, dürfte von jedem, der sich mit Problemen der Kreislaufregulation, des Blutersatzes und der Schockbehandlung beschäftigt, mit Interesse gelesen werden

U BUCHER, *Bern*

Varia

Karger Medical and Scientific Publishers announce a new section on 'Inborn Errors of Metabolism in Man', to appear regularly under a special heading in the journal *Human Heredity* Articles are invited related to the metabolic aspects of the various hereditary errors of metabolism in man The editorial committee includes ANDRE DE VRIES (Petah Tikva), R W E WATTS (London), S SEGAL (Philadelphia) and ODED SPERLING (Petah Tikva) Papers should be prepared according to the rules for the preparation of manuscripts for the Karger journals, and not exceed six printed (journal print) pages Papers should be submitted to the chief editor of this new section ANDRE DE VRIES, Beilinson Medical Center, PO Box 85 *Petah Tikva* (Israel)

Acta haemat. 55: 193–199 (1976)

Intravascular Fate of Granulocytes Administered by Granulocyte Transfusions[1]

G. Meuret, H. J. Senn, V. de Fliedner and M. Fopp

Department of Internal Medicine, Section of Hematology and Oncology, Kantonsspital, St. Gallen

Key Words. Autotransfusion of granulocytes · Granulocyte transfusion · Leukocyte kinetics

Abstract. Granulocytes were harvested from hematologically normal individuals using continuous flow centrifugation (CFC) or filtration leukapheresis (FL). The isolated granulocytes were labeled *in vitro* by ^{3}H-diisopropyl fluorophosphate (^{3}H DFP) and autotransfused. Their intravascular fate was analyzed by autoradiography. Immediately after autotransfusion the majority of granulocytes administered, collected in the marginal granulocyte pool. Margination was particularly prominent in granulocytes isolated by FL. The distribution of transfused granulocytes between the circulating and the marginal granulocyte pool showed wide and irregular fluctuations in time. Margination of transfused granulocytes was counterbalanced and its fluctuation between the two intravascular pools was stabilized by prednisone treatment. The transit of transfused granulocytes from blood to tissue seemed to be governed by a random process, the half-disappearance time being either normal or prolonged. Compatible granulocytes administered to hematologically normal recipients circulated for at least 20 h.

Granulocyte transfusion is applied increasingly in the management of life-threatening infections during severe neutropenia [10]. The cells administered with these transfusions are generally collected from normal donors using continuous flow centrifugation (CFC) or filtration leukapheresis (FL). *In vitro* studies of granulocytes harvested by these methods did not reveal any relevant morphological or functional defects [3, 7]. The purpose of the present paper is to analyze their *in vivo* behavior by cell kinetic studies using *in vitro* ^{3}H-DFP labeling, autotransfusion, and autoradiography.

[1] Research supported by Swiss Cancer League grant No. SKL FOR 034 AK 73 (1).

Book Review · Buchbesprechung · Livre nouveau

J. M. UNSELD: **Blutersatz durch stromafreie Hämoglobinlösung.** Ergebnisse tierexperimenteller Untersuchungen. Springer, Berlin 1974. VII + 90 pp., 17 fig. DM 32.–/US$ 13.10.

Der Autor legt die vollständige Dokumentation über seine Untersuchungen mit einer stromafreien Hämoglobinlösung am normovolämischen und hypovolämischen Zwergschwein vor. Zum Vergleich diente eine Albuminlösung bzw. bei den durch Blutung in den hämorrhagischen Schock versetzten Tieren die Retransfusion des Blutes. Es wurde das Verhalten verschiedener Kreislaufgrössen, des O_2-Transportvermögens, der O_2-Aufnahme und der Nierenfunktion untersucht. In Bestätigung früherer Befunde von RABINER erwies sich die Hämoglobinlösung nicht als nephrotoxisch. Im hämorrhagischen Schock hatte sie kurzfristig eine dem Vollblut ähnliche, den Plasmaexpandern in bezug auf O_2-Transport überlegene Wirkung. Durch die – speziesabhängige – kurze Halbwertszeit für das Verschwinden aus dem Plasma (beim Schwein ca. $3^1/_2$ h) ist die Anwendbarkeit allerdings beschränkt. Die Befunde werden eingehend diskutiert, wobei jedoch die neueste Literatur keine Berücksichtigung findet, da die Arbeit offenbar schon 1972 abgeschlossen worden ist. Aus der Nichtberücksichtigung wichtiger älterer Arbeiten – z. B. derjenigen von BUNN *et al.* – erklärt sich wahrscheinlich die etwas einseitige Betrachtungsweise. So halten die Angaben über die Elimination der Hämoglobinlösung nicht jeder Kritik stand. Ähnliches gilt für die Bemerkungen über die Sauerstoffabgabe. Das kleine Buch, dem eine kurze englische Zusammenfassung beigegeben ist, dürfte von jedem, der sich mit Problemen der Kreislaufregulation, des Blutersatzes und der Schockbehandlung beschäftigt, mit Interesse gelesen werden.

U. BUCHER, *Bern*

Varia

Karger Medical and Scientific Publishers announce a new section on 'Inborn Errors of Metabolism in Man', to appear regularly under a special heading in the journal *Human Heredity*. Articles are invited related to the metabolic aspects of the various hereditary errors of metabolism in man. The editorial committee includes ANDRE DE VRIES (Petah Tikva), R. W. E. WATTS (London), S. SEGAL (Philadelphia) and ODED SPERLING (Petah Tikva). Papers should be prepared according to the rules for the preparation of manuscripts for the Karger journals, and not exceed six printed (journal print) pages. Papers should be submitted to the chief editor of this new section: ANDRE DE VRIES, Beilinson Medical Center, PO Box 85, *Petah Tikva* (Israel).

Acta haemat. 55: 193–198 (1976)

Intravascular Fate of Granulocytes Administered by Granulocyte Transfusions[1]

G. Meuret, H. J. Senn, V. de Fliedner and M. Fopp

Department of Internal Medicine, Section of Hematology and Oncology, Kantonsspital St. Gallen

Key Words. Autotransfusion of granulocytes · Granulocyte transfusion · Leukocyte kinetics

Abstract. Granulocytes were harvested from hematologically normal individuals using continuous flow centrifugation (CFC) or filtration leukapheresis (FL). The isolated granulocytes were labeled *in vitro* by ^{3}H-diisopropyl fluorophosphate (^{3}H DFP) and autotransfused. Their intravascular fate was analyzed by autoradiography. Immediately after autotransfusion the majority of granulocytes administered collected in the marginal granulocyte pool. Margination was particularly prominent in granulocytes isolated by FL. The distribution of transfused granulocytes between the circulating and the marginal granulocyte pool showed wide and irregular fluctuations in time. Margination of transfused granulocytes was counterbalanced and its fluctuation between the two intravascular pools was stabilized by prednisone treatment. The transit of transfused granulocytes from blood to tissue seemed to be governed by a random process, the half-disappearance time being either normal or prolonged. Compatible granulocytes administered to hematologically normal recipients circulated for at least 20 h.

Granulocyte transfusion is applied increasingly in the management of life threatening infections during severe neutropenia [10]. The cells administered with these transfusions are generally collected from normal donors using continuous flow centrifugation (CFC) or filtration leukapheresis (FL). *In vitro* studies of granulocytes harvested by these methods did not reveal any relevant morphological or functional defects [3, 7]. The purpose of the present paper is to analyze their *in vivo* behavior by cell kinetic studies using *in vitro* ^{3}H DFP labeling, autotransfusion, and autoradiography.

[1] Research supported by Swiss Cancer League, grant No. SKL FOR 034.AK 73 (1).

J. M. Unseld: **Blutersatz durch stromafreie Hämoglobinlösung.** Ergebnisse tierexperimenteller Untersuchungen. Springer, Berlin 1974. VII + 90 pp., 17 fig. DM 32.–/US$ 13.10.

Der Autor legt die vollständige Dokumentation über seine Untersuchungen mit einer stromafreien Hämoglobinlösung am normovolämischen und hypovolämischen Zwergschwein vor. Zum Vergleich diente eine Albuminlösung bzw. bei den durch Blutung in den hämorrhagischen Schock versetzten Tieren die Retransfusion des Blutes. Es wurde das Verhalten verschiedener Kreislaufgrössen, des O_2-Transportvermögens, der O_2-Aufnahme und der Nierenfunktion untersucht. In Bestätigung früherer Befunde von Rabiner erwies sich die Hämoglobinlösung nicht als nephrotoxisch. Im hämorrhagischen Schock hatte sie kurzfristig eine dem Vollblut ähnliche, den Plasmaexpandern in bezug auf O_2-Transport überlegene Wirkung. Durch die – speziesabhängige – kurze Halbwertszeit für das Verschwinden aus dem Plasma (beim Schwein ca. $3^1/_2$ h) ist die Anwendbarkeit allerdings beschränkt. Die Befunde werden eingehend diskutiert, wobei jedoch die neueste Literatur keine Berücksichtigung findet, da die Arbeit offenbar schon 1972 abgeschlossen worden ist. Aus der Nichtberücksichtigung wichtiger älterer Arbeiten – z. B. derjenigen von Bunn *et al.* – erklärt sich wahrscheinlich die etwas einseitige Betrachtungsweise. So halten die Angaben über die Elimination der Hämoglobinlösung nicht jeder Kritik stand. Ähnliches gilt für die Bemerkungen über die Sauerstoffabgabe. Das kleine Buch, dem eine kurze englische Zusammenfassung beigegeben ist, dürfte von jedem, der sich mit Problemen der Kreislaufregulation, des Blutersatzes und der Schockbehandlung beschäftigt, mit Interesse gelesen werden.

U. Bucher, *Bern*

Varia

Karger Medical and Scientific Publishers announce a new section on 'Inborn Errors of Metabolism in Man', to appear regularly under a special heading in the journal *Human Heredity*. Articles are invited related to the metabolic aspects of the various hereditary errors of metabolism in man. The editorial committee includes André de Vries (Petah Tikva), R. W. E. Watts (London), S. Segal (Philadelphia) and Oded Sperling (Petah Tikva). Papers should be prepared according to the rules for the preparation of manuscripts for the Karger journals, and not exceed six printed (journal print) pages. Papers should be submitted to the chief editor of this new section: André de Vries, Beilinson Medical Center, PO Box 85, *Petah Tikva* (Israel).

Acta haemat. 55: 193–198 (1976)

Intravascular Fate of Granulocytes Administered by Granulocyte Transfusions[1]

G. MEURET, H. J. SENN, V. DE FLIEDNER and M. FOPP

Department of Internal Medicine, Section of Hematology and Oncology, Kantonsspital, St. Gallen

Key Words. Autotransfusion of granulocytes · Granulocyte transfusion · Leukocyte kinetics

Abstract. Granulocytes were harvested from hematologically normal individuals using continuous flow centrifugation (CFC) or filtration leukapheresis (FL). The isolated granulocytes were labeled *in vitro* by ^3H-diisopropyl fluorophosphate (^3H-DFP) and autotransfused. Their intravascular fate was analyzed by autoradiography. Immediately after autotransfusion the majority of granulocytes administered collected in the marginal granulocyte pool. Margination was particularly prominent in granulocytes isolated by FL. The distribution of transfused granulocytes between the circulating and the marginal granulocyte pool showed wide and irregular fluctuations in time. Margination of transfused granulocytes was counterbalanced and its fluctuation between the two intravascular pools was stabilized by prednisone treatment. The transit of transfused granulocytes from blood to tissue seemed to be governed by a random process, the half-disappearance time being either normal or prolonged. Compatible granulocytes administered to hematologically normal recipients circulated for at least 20 h.

Granulocyte transfusion is applied increasingly in the management of life-threatening infections during severe neutropenia [10]. The cells administered with these transfusions are generally collected from normal donors using continuous flow centrifugation (CFC) or filtration leukapheresis (FL). *In vitro* studies of granulocytes harvested by these methods did not reveal any relevant morphological or functional defects [3, 7]. The purpose of the present paper is to analyze their *in vivo* behavior by cell kinetic studies using *in vitro* ^3H-DFP labeling, autotransfusion, and autoradiography.

[1] Research supported by Swiss Cancer League, grant No. SKL FOR 034 AK 73 (1).

Patients and Methods

The studies were carried out in 10 hematologically normal patients with malignant diseases. They all gave informed consent. Four subjects were treated with 3×10 mg prednisone per day, given orally, for the duration of the cell kinetic studies except for 1 patient, F.E. (fig. 1), who received prednisone only during the first day of the investigation. Granulocytes were collected either by the Aminco continuous flow centrifuge (American Instrument Co., Silver Spring, Md.) or by the repetitive filtration elution leukapheresis as described earlier [4]. Heparine (with FL) or heparine plus ACD (with CFC) was added to the donors' blood during cell separation while polymers such as hydroxyethylstarch or plasmagel were not applied with CFC.

The cell kinetic methods used in the present study have already been described in detail [9, 11]. In short, collected granulocytes were labeled *in vitro* by 500 μCi ^{3}H diisopropyl fluorophosphate (^{3}H DFP, specific activity 5 Ci/mM) and autotransfused within 10 min. Subsequently venous blood samples were taken at suitable intervals for preparation of concentrated leukocyte smears which, after processing by autoradiography, were submitted to microscopic analysis. Granulocyte labeling indices were determined by scanning 3,000 granulocytes per sample. The circulating granulocyte pool (CGP) of the recipients was calculated from blood volume using the formula of Baker *et al.* [1] and the mean neutrophil blood count during the study. The number of transfused granulocytes in CGP after completion of autotransfusion resulted from CGP×LI/100 (where LI = 5 min posttransfusion). The number of transfused granulocytes marginating 5 min after autotransfusion was the difference between the total number of transfused labeled granulocytes and the number of circulating transfused granulocytes.

Results

^{3}H-DFP labeling indices of transfused granulocytes ranged between 94 and 100%. The following numbers of labeled granulocytes (×10^9) were administered to the patients: H.H. 5.9, S.P. 6.6, F.I. 2.1, W.A. 3.2, F.E. 1.1, E.X. 2.5, G.C. 1.9, B.M. 0.9, E.A. 6.5, and L.W. 6.9. In patients not being treated by prednisone the majority of the transfused granulocytes disappeared rapidly from the circulating blood. 5 min after the autotransfusion the majority of the transfused granulocytes were located in the marginal granulocyte pool (fig. 1, 2).

With granulocytes isolated by FL, margination was more pronounced than with those isolated by CFC. In patients not being treated with prednisone, granulocyte-labeling indices scattered irregularly during the course of the investigation except in patient F.I. In this latter case, labeled granulocytes disappeared exponentially from the circulating blood with a half-

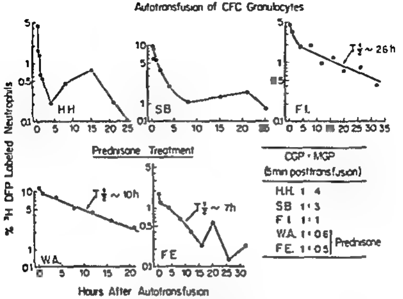


Fig 1 Intravascular kinetics of neutrophilic granulocytes isolated by CFC, labeled *in vitro* with ^{3}H DFP and autotransfused to hematologically normal individuals. CGP:MGP indicates the distribution of the transfused granulocytes between the circulating and the marginal granulocyte pool of the recipients

disappearance time of about 26 h (fig 1) Prednisone treatment elicited a shift of transfused granulocytes to the circulating pool and caused the time course of granulocyte-labeling indices to be more regular In 3 of 4 patients, labeling indices fell in an exponential fashion, the half-times being 7, 10 and 13 h, respectively (fig 1, 2) In patient F E, scattering of granulocyte-labeling indices appeared during the second day of the study (fig 1) when prednisone treatment was discontinued In all studies labeled granulocytes administered by the granulocyte transfusion could be detected in the blood of the recipients during a period of at least 20 h

Discussion

Interpretation of the results may be difficult due to possible perturbations in the steady state equilibrium of granulocyte kinetics provoked by

Patients and Methods

The studies were carried out in 10 hematologically normal patients with malignant diseases. They all gave informed consent. Four subjects were treated with 3×10 mg prednisone per day, given orally, for the duration of the cell kinetic studies except for 1 patient, F.E. (fig. 1) who received prednisone only during the first day of the investigation. Granulocytes were collected either by the Aminco continuous flow centrifuge (American Instrument Co., Silver Spring, Md.) or by the repetitive filtration elution leukapheresis as described earlier [4]. Heparine (with FL) or heparine plus ACD (with CFC) was added to the donors' blood during cell separation while polymers such as hydroxyethylstarch or plasmagel were not applied with CFC.

The cell kinetic methods used in the present study have already been described in detail [9, 11]. In short, collected granulocytes were labeled *in vitro* by 500 μCi ^{3}H diisopropyl fluorophosphate (^{3}H DFP, specific activity 5 Ci/mM) and autotransfused within 10 min. Subsequently venous blood samples were taken at suitable intervals for preparation of concentrated leukocyte smears which, after processing by autoradiography, were submitted to microscopic analysis. Granulocyte labeling indices were determined by scanning 3,000 granulocytes per sample. The circulating granulocyte pool (CGP) of the recipients was calculated from blood volume using the formula of Baker *et al.* [1] and the mean neutrophil blood count during the study. The number of transfused granulocytes in CGP after completion of autotransfusion resulted from CGP×LI/100 (where LI = 5 min posttransfusion). The number of transfused granulocytes marginating 5 min after autotransfusion was the difference between the total number of transfused labeled granulocytes and the number of circulating transfused granulocytes.

Results

^{3}H-DFP labeling indices of transfused granulocytes ranged between 94 and 100%. The following numbers of labeled granulocytes (×10^9) were administered to the patients: H.H. 5.9, S.P. 6.6, F.I. 2.1, W.A. 3.2, F.E. 1.1, E.X. 2.5, G.C. 1.9, B.M. 0.9, E.A. 6.5, and L.W. 6.9. In patients not being treated by prednisone the majority of the transfused granulocytes disappeared rapidly from the circulating blood. 5 min after the autotransfusion the majority of the transfused granulocytes were located in the marginal granulocyte pool (fig. 1, 2).

With granulocytes isolated by FL, margination was more pronounced than with those isolated by CFC. In patients not being treated with prednisone, granulocyte-labeling indices scattered irregularly during the course of the investigation except in patient F.I. In this latter case, labeled granulocytes disappeared exponentially from the circulating blood with a half-

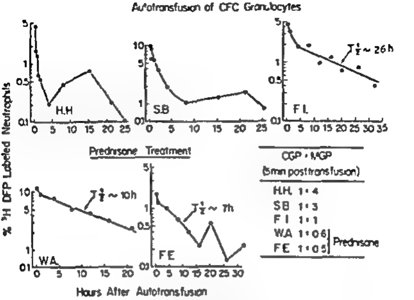


Fig 1 Intravascular kinetics of neutrophilic granulocytes isolated by CFC, labeled *in vitro* with ³H DFP and autotransfused to hematologically normal individuals CGP MGP indicates the distribution of the transfused granulocytes between the circulating and the marginal granulocyte pool of the recipients

disappearance time of about 26 h (fig 1) Prednisone treatment elicited a shift of transfused granulocytes to the circulating pool and caused the time course of granulocyte-labeling indices to be more regular In 3 of 4 patients, labeling indices fell in an exponential fashion, the half times being 7, 10 and 13 h, respectively (fig 1, 2) In patient F. E., scattering of granulocyte-labeling indices appeared during the second day of the study (fig 1) when prednisone treatment was discontinued In all studies labeled granulocytes administered by the granulocyte transfusion could be detected in the blood of the recipients during a period of at least 20 h

Discussion

Interpretation of the results may be difficult due to possible perturbations in the steady state equilibrium of granulocyte kinetics provoked by

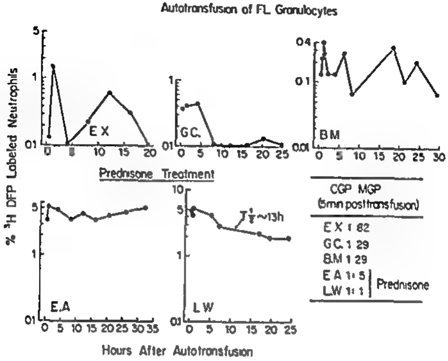


Fig 2 Intravascular kinetics of neutrophilic granulocytes isolated by FL and autotransfused See legend of figure 1

the cell separation procedure However, to minimize this perturbation only relatively small amounts of granulocytes were harvested by CFC or FL The proportion of transfused granulocytes being detectable in circulation some minutes after completion of autotransfusion was relatively small Two arguments are in favor of the conclusion that the majority of the granulocytes resided in the marginal granulocyte pool and were not victim to phagocytosis by the reticuloendothelial system Firstly, in some of the patients the time course of granulocyte-labeling indices was irregular including high peaks This phenomenon seems to indicate a disorderly fluctuation of transfused granulocytes between CGP and MGP Secondly, prednisone *treatment effected considerable enlargement of the proportion* of transfused granulocytes in the CGP These considerations seem to justify the following interpretations and conclusions

(1) Granulocytes administered by granulocyte transfusions tend to accumulate in MGP Margination is more pronounced with granulocytes isolated by FL than with those isolated by CFC The difference may arise from cell interactions with the nylon fibers of the filters during cell sepa-

ration Margination of transfused granulocytes is probably the explanation for the low recovery rates reported in the literature [5, 6]

(2) The distribution equilibrium of transfused granulocytes between the two intravascular pools is unstable

(3) Margination of transfused granulocytes was effectively counterbalanced by the application of prednisone which, in addition, stabilized the distribution equilibrium of these cells between the two intravascular pools

(4) In some patients, irregular cell fluctuations between CGP and MGP did not allow for determination of the cells' disappearance curves In others, the curves followed a decaying exponential component, indicating a random transit of transfused granulocytes from the vessels into the tissue In these latter cases the half disappearance times were either in the range of normal granulocytes or were prolonged (normal range of the method 6 1–10 5 h mean 7 6 h [5]) Similar half-disappearance times were observed when allogenic neutrophils were transfused to neutropenic recipients with infections [4] The results of our cell kinetic studies in men are basically in accordance with similar observations in dogs obtained by BUCKNER *et al* [2]

(5) In hematologically normal recipients compatible granulocytes administered with granulocyte transfusions circulate for at least 20 h

In patients with agranulocytosis quantitative collections of neutrophils in the exudate of acute inflammations were carried out before and after transfusion of compatible granulocytes [4] These studies evidenced rapid accumulation and exudation of transfused granulocytes at the site of the inflammatory reaction This behavior may explain why relatively small numbers of granulocytes administered by granulocyte transfusions have proven effective in the treatment of septicemia in granulocytopenic patients [6 8]

References

1 BAKER R J ROZOLL D D., and MEYER K. The use of surface area as a basis for establishing normal blood volume Surgery Gynec Obstet *104* 183 (1957)

2 BUCKNER D GRAW R G EISEL, R J, HENDERSON E. S., and PERRY S Leucapheresis by continuous flow centrifugation (CFC) in patients with chronic myelocytic leukemia (CML) Blood *33* 353 (1969)

3 DEBELLAK K M EPSTEIN R B., and ANDERSEN B R Granulocyte transfusions in leukopenic dogs *In vivo* and *In vitro* function of granulocytes obtained by continuous flow filtration leucapheresis Blood *43* 757 (1974)

4 FLIEDNER V DE, MEURET, G, and SENN, H J Normal granulocyte collection with a modified repetitive cycle filtration leucapheresis Blut *29* 265 (1974)
5 GRAW, R G, HERZIG, G P, EISEL, R J, and PERRY, S Leukocyte and platelet collection from normal donors with the continuous flow blood cell separator Transfusion Philad *11* 94 (1971)
6 GRAW, R G, HERZIG, G P, PERRY, S, and HENDERSON E S Normal granulocyte transfusion therapy New Engl J Med *287* 367 (1972)
7 HARRIS, M B, DJERASSI I, SCHWARTZ, E, and ROOT, R K Polymorphonuclear leukocytes prepared by continuous flow filtration leukapheresis viability and function Blood *44* 707 (1974)
8 HIGBY, D J, YATES J W, HENDERSON, E S, and HOLLAND J F Filtration leukapheresis for granulocyte transfusion therapy New Engl J Med *292* 761 (1975)
9 MEURET, G and FLIEDNER T M Neutrophil and monocyte kinetics in a case of cyclic neutropenia *Blood 43 565 (1974)*
10 MEURET, G und SENN H J Granulozytentransfusion Ein Überblick Schweiz med Wschr *105* 225 (1975)
11 MEURET, G, HOFFMANN, G, FLIEDNER, T M, RAU, M, OEHL, S, WALZ, R and KLEIN WISENBERG, A Neutrophil kinetics in man Studies using autotransfusion of ³H DFP labeled blood cells and autoradiography Blut *26* 97 (1973)

PD Dr G MEURET, Medizinische Klinik C, Kantonsspital, *CH-9006 St Gallen* (Switzerland)

Acta haemat 55 199-204 (1976)

Cytochemical Leukocyte Reactions in Normal Children

J. Corberand

Laboratoire d'Hematologie, CHU de Rangueil, Toulouse

Key Words Alkaline phosphatases · Cytochemistry · Esterases · Leukocyte metabolism · Myeloperoxydase · Periodic acid Schiff reaction · Sudan Black reaction

Abstract Five cytochemical reactions in current use (MPO, PAS, LAP, SB and EST) were operated on 180 children aged 1 month to 6 years, divided into three age groups (less than 12 months, 1-3 years and 3-6 years). No significant differences are apparent from one group to another, and the mean values are identical with those of adults. On the other hand, certain variations are evident in comparison with the scores of newborn children: important decrease in LAP, increase in SB, EST and PAS. These tests should be done in investigations of inborn metabolic errors, chromosome anomalies, blood diseases, and defects in defense mechanisms.

Cytochemical reactions are becoming increasingly more important for the study of blood cells. They are helpful for diagnosis of blood diseases and, above all, for classifying acute leukemia. For research purposes, metabolic changes which occur in various pathological states can be approached from this angle, for example, hereditary diseases. These tests can be carried out with relative ease on some drops of capillary blood. Such study would seem of particular interest in pediatric practice.

Cytochemical leukocyte studies are numerous and deal primarily with adults, and the norm is sometimes uncertain. We feel it useful to set up normal values in children and to consider the resulting variations which appear during the first years of life. In a previous paper, we published the results for normal newborn children [1]. The following study is concerned with children between the ages of a few weeks and 6 years.

Materials and Methods

We studied 180 children aged between a few weeks and 6 years They were all considered as normal, i e free of any clinically detectable illness They were divided into three groups

Group 1 40 babies were chosen at random from the Pediatric Center of the Maternity Hospital They were 18 boys and 22 girls, all over 1 month and less than 12 months old

Group 2 A blood sample was taken from 57 children (34 boys and 23 girls aged 1–3 years, at a nursery in town

Group 3 83 samples were obtained from children aged 3–6 years who came from a kindergarden They were 43 boys and 40 girls

This division into three age groups did not depend on known or suspected physiological variations, it was simply a result of the method of recruitment

Five cytochemical tests were used They were carried out on blood smears obtained from finger prick samples At the same time, a hemogram was performed For each cytochemical reaction a score based on 100 cells was established and grades assigned according to the intensity of the reaction

Myeloperoxydase (MPO) GRAHAM KNOLL technique [2] modified by shortening the reaction time (75 sec instead of 5 min) The score ranged from 0 to 300

Leukocyte alkaline phosphatase (LAP) α Naphtyl phosphate technique followed by diazoreaction according to a modification in the Gomori method [3] The Kaplow score had a theoretical variation from 0 to 400

Non specific esterase (EST) WACHSTEIN and WOLF technique [4] modified by GROZDEA *et al* [5] We chose a score varying between 0 and 300

Polysaccharides by periodic acid Schiff reaction (PAS) McManus technique modified by lengthening the reaction time and omitting rinsing in sulfurous medium Two scores were used 0–300 for polymorphonuclear and 0–300 for lymphocytes

Lipids by Sudan Black (SB) Technique of HAYOHE *et al* [6] The score varied from 0 to 400

Results

The results of the 5 reactions in each age group are shown in table I The number of subjects studied in each group varies from reaction to reaction, this is mainly due to technical problems (reaction failure, illegibility due to the poor quality of the smear or to morphological leukocyte changes)

Comments

Blood leukocytes play a vital role in specific or nonspecific defense of the body against pathogenic agents These cells use general cellular meta-

Table 1 Results of cytochemical leukocyte reactions in children of three age groups

Reactions[1]	Number of subjects	Grading 0	1	2	3	4	Scores	Standard error
Children less than 12 months old								
SB	41	0.00	0.02	4.61	68.88	26.49	321.83	19.16
MPO	33	0.45	5.88	85.24	6.91		197.09	19.95
PMN PAS	44	2.43	20.91	71.50	4.77		178.23	32.78
LAP	44	29.14	25.18	24.34	20.34	1.00	138.89	46.49
EST	20	0.31	29.72	55.72	14.33		180.00	29.51
L-PAS	44	86.[illegible]	8.73	3.84	0.61		18.25	10.39
Children aged 1–3 years								
SB	53	0.00	0.00	1.71	69.92	28.28	326.49	13.95
MPO	41	0.05	2.12	80.22	17.61		215.39	14.38
PMN PAS	53	0.00	17.53	77.87	4.58		187.02	15.39
LAP	52	15.15	21.17	29.54	32.27	1.87	184.52	50.97
EST	20	0.00	24.25	29.50	16.25		192.00	27.59
L PAS	53	80.40	15.58	3.89	0.13		23.75	14.95
Children aged 3–6 years								
SB	82	0.00	0.02	0.62	74.02	25.45	325.15	12.72
MPO	80	0.22	6.04	86.55	7.19		200.71	15.67
PMN PAS	[illegible]	0.01	14.54	81.28	3.67		188.11	17.23
LAP	75	22.09	21.83	25.31	29.20	1.49	166.24	54.98
EST	20	0.35	29.35	57.10	13.20		183.15	33.14
L PAS	82	80.93	16.73	2.26	0.09		21.50	9.87

[1] SB = Sudan Black MPO = myeloperoxidase PMN PAS = polymorphonuclear periodic acid Schiff reaction LAP = leukocyte alkaline phosphatase, EST = non specific esterase L PAS = lymphocyte periodic acid Schiff reaction

bolic ways and it may be interesting to study them for research purposes in genetic metabolic diseases [7].

The five tests used in this work were chosen for their practical interest, and particularly for cytological diagnosis of malignant blood diseases. They are quite easily carried out and enable one to approach metabolic cell activity. Thus MPO plays a fundamental role in bactericidal activity of phagocytes [8]. Lipids, some of which are shown by Sudan Black,

Table II Comparison of mean scores for the five cytochemical reactions in full born babies and adults

Reactions[1]	Age group				adults		
	newborns	< 12 months	1–3 years	3–6 years	males		females
SB	299	322	326	325	–		–
MPO	195	197	215	201	215		218 5
PMN PAS	177	178	187	188		168 50	
LAP	220	139	184	166	105 5		155
EST	141	180	192	183	–		–
L-PAS	9	18	24	22		22 5	

[1] For abbreviations, see table I

make up part of the cellular membrane and constitute an important reserve of energy, together with MPO, they may play a part in defense against bacterial infection The PAS positive component of granulocytes is essentially glycogen which, in its intracytoplasmic accumulation, constitutes an important reserve of energy for phagocytosis On the other hand, the quantities contained in lymphocytes are too small to be shown by PAS reaction, this explains their very low scores The precise role of esterases remains unclear, they may take part in metabolic events occurring during phagocytosis [9] LAP, of which several isoenzymes are known, participates in glucose and lipid metabolism and in cell division Their production is controlled by sex hormones [10] and their level increases during infection This would seem to show the interest of performing these three cytochemical reactions (MPO, PAS, LAP) as part of an investigation of nonspecific cellular defense mechanisms As these elements are under genetic control, the study of cytochemical reactions would seem justified for hereditary chromosomal anomalies

No significant variation can be seen between age groups in these five tests, however, the highest mean values are always found in the age group 1–3 years The different score levels are similar to these of adults for PAS, LAP and MPO On the other hand, the results differ from those of full-born babies (table II) Three types of evolution can be noted during the first month (1) an important decline in LAP, (2) no PMN-PAS or MPO variation, (3) an increase in SB, EST and L-PAS

The following remarks may be made about these results. The LAP variations may depend on neonatal hormonal changes. The absence of modifications in granulocyte PAS and MPO would seem to suggest that leukocyte (functional) activity is identical whatever the age. As for the increase of PAS in lymphocytes between the time of birth and 3 years, the only possible parallel is with the acquisition of immune information which is highest at this age.

The difficulty arises for the comparison of our results with others, as very few publications cover these problems. To our knowledge, the only precise work is that of KATATA [11] on LAP. In any case, this author has not used the same scoring as we did, alone the general aspect of our results may be compared: he found the same increase in LAP at birth and lower scores for infants of 9–10 months, after which the scores rose again up to a slightly higher level than those of adults.

The use of the cytochemical test on leukocytes offers two points of interest. For basic blood research, they give information about certain aspects of cell metabolism and their study in chromosome anomalies can clarify ideas about certain substrata and enzyme regulation. In practice, they can be of particular help for the diagnosis of some inborn metabolic errors, of blood diseases such as acute leukemia and susceptibility to infection. This work enables normal scores to be set up for the five reactions used in children during the first 6 years, which allows a better interpretation of the results in children.

References

1 CORBERAND, J.: Cytochemical leukocyte reactions in normal newborn infants. Biol. Neonate 22: 280–285 (1973).

2 GRAHAM-KNOLL: Réactions pour le diagnostic général; in Planches d'hématologie Sandoz (Sandoz, Basel 1972).

3 LEJEUNE, F.: Techniques cytologiques utiles en pratique courante; in Cytologie pratique du sang et des organes hématopoïétiques (Doin Derain, Paris 1966).

4 WACHSTEIN, M. and WOLF, G.: The histochemical demonstration of esterases activity in human blood and bone marrow smears. J. Histochem. Cytochem. 6: 457 (1958).

5 *GROZDEA, J.; COLOMBIES, P. et DUCOS, J.:* Etude cytochimique et chromosomes en-dehors des hémopathies. Nouv. Revue fr. Hémat. 9: 722 (1969).

6 HAYHOE, F.; QUAGLINO, D., and DOLL, R.: Cytology and cytochemistry of acute leukemias, p. 23 (Her Majesty's Stationary Office, London 1964).

7 TRUBOWITZ, S.; KIRMAN, D., and MASEK, B.: The leukocyte *alkaline phosphatase* in mongolism. Lancet *ii:* 486 (1962).

8 KLEBANOFF, S J Myeloperoxidase mediated and microbial systems and their role in leukocyte function, in SCHULTZ Biochemistry of the phagocytic process (North Holland, Amsterdam 1970)
9 FLANDRIN, G et DANIEL, M T Cytochimie des esterases en hématologie Path Biol, Paris *19* 577 (1971)
10 RAY, P K and PINKERTON, P H Leukocyte alkaline phosphatase The effects of age and sex Acta haemat *42* 18 (1969)
11 KATATA, Y Cytochemical studies on leukocyte alkaline phosphatase by naphtol AS MX phosphate fast blue RR staining method from the pediatric aspect Hiroshima J med Sci *14* 129 (1965)

Dr J CORBERAND, Laboratoire d'Hématologie, CHV Rangueil, chemin du Vallon, *F–31052 Toulouse Cédex* (France)

Acta haemat 55 205-215 (1976)

Elektronenmikroskopische Lokalisation von Thiaminpyrophosphatase und Nucleosiddiphosphatase in Lymphozyten

ARMIN E. FRIESS

Institut für Anatomie, Fachbereich Biologie und vorklinische Medizin, Universität Regensburg, Regensburg

Key Words Lymphocyte enzymes · Phosphatases · Ultrahistochemistry

Abstract In ductus thoracicus lymphocytes of the rat the localization of specific phosphatases was studied by means of ultrahistochemistry. Thiaminepyrophosphatase was found in the outer lamellae of the Golgi field as well as in lysosomes, whereas nucleosiddiphosphatases could be localized on the plasma membrane at low pH. These findings are correlated with the phaenotype of B- and T-lymphocytes. Their functional significance is discussed.

Lichtmikroskopische enzymhistochemische Untersuchungen an Zellen der lymphatischen Organe von Mensch und zahlreichen Säugern [17, 18, 35] lassen erkennen, dass der Gehalt an phosphatspaltenden Enzymen sowohl spezies- als auch organabhängig ist. In der menschlichen Milz zeichnen sich T-zellabhängige und B-zellabhängige Regionen durch den Besitz charakteristischer Enzyme aus [24]. Periphere Lymphozyten scheinen dagegen nur sehr spärlich mit nachweisbaren Enzymaktivitäten ausgestattet zu sein [1, 5, 7, 17]. Während beim Meerschweinchen Thymozyten in der Thymusrinde eine hohe Aktivität der alkalischen Phosphatasen besitzen, konnte dieses Enzym in peripheren T-Lymphozyten nicht mehr nachgewiesen werden [17, 32].

Eine deutliche Abhängigkeit vom Differenzierungsstadium der Lymphozyten weist auch die ATPase auf. Plasmoblasten sind durch eine höhere Enzymaktivität gekennzeichnet als Plasmazellen und nicht aktivierte B-Lymphozyten [12, 28, 37]. Spezifische Phosphatasen, wie die Thiaminpyrophosphatase (TTPase) oder Nucleosiddiphosphatasen (Inosiddiphosphatase = IDPase, Uridindiphosphatase = UDPase, Guanosidinphosphatase = GDPase) sind eng mit dem Golgi-Feld bzw. mit dem Plas-

malemm assoziiert. An stimulierten Lymphozyten aus dem Lymphknoten des Meerschweinchens konnte eine TPPase-Aktivität in den inneren Lamellen des Golgi-Feldes nachgewiesen werden [4]. Frühere Untersuchungen an Ductus-thoracicus(DT)-Lymphozyten des Hundes haben eine Enzymaktivität vornehmlich an der konvexen Seite der Dictiosomen ergeben [11]. Da das Golgi-Feld eine wichtige Rolle bei der Glykoproteinsynthese spielt und Membranen des Golgi-Feldes teils als Komponenten in die Zellmembran ausgeschleust werden, kommt dem Golgi-Apparat kleiner Lymphozyten mit Hinblick auf ihre Oberflächenspezialisierung eine wesentliche Bedeutung zu.

Material und Methode

Zur Untersuchung gelangten Lymphozyten aus dem DT der Ratte. Die DT-Drainage wurde nach BOLLMANN *et al.* [6] durchgeführt. Die Lymphozyten wurden in 1,5% Glutaraldehyd in 0,1 M Cacodylatpuffer (380 mosm) 20 min bei 4 °C fixiert, anschliessend zentrifugiert und das Pellet zweimal in Puffer gewaschen. Der Nachweis der TPPase bzw. NDPase erfolgte im Medium nach NOVIKOFF und GOLDFISCHER [26] für 2 h bei 37 °C. Medium: TPP 2 mM bzw. UDP, MnCl 5 mM, Trismaleatpuffer 0,2 M, pH 5,0–7,5, Pb $(NO_3)_2$ 3,6 mM. Das Medium wurde vor Gebrauch auf 37 °C erwärmt und filtriert. Uranylcitrat in 0,01 M Konzentration wirkte als Inhibitor. Nach der Inkubation wurde das Pellet in 0,1 M Cacodylatpuffer gewaschen, mit 1% gepufferter OsO_4 nachfixiert, über Aceton entwässert und in Epon/Araldit-Gemisch eingebettet. Die Ultradünnschnitte wurden teilweise mit Uranylacetat nachkontrastiert.

Befunde

In kleinen Lymphozyten aus dem DT beschränkt sich der Nachweis eines TPP-spaltenden Enzyms im wesentlichen auf das Golgi-Feld. Zusätzlich weisen Lysosomen Reaktionsprodukte auf (Abb. 1). In der Regel sind die äusseren konvexen 1–2 Golgi-Zisternen markiert (Abb. 2a), während die Innenzisternen des ohnehin schmalen Golgi-Feldes frei bleiben. Der Niederschlag ist aber auch innerhalb der markierten Membranstapel nicht gleichmässig verteilt. Häufig wird sehr dickes Präzipitat in der äussersten Zisterne gefunden oder in den kolbenförmig erweiterten Endabschnitten der einzelnen Membranabschnitte (Abb. 2b). Andere Teile des Golgi-Feldes weisen sehr schwaches und locker verteiltes Reaktionsprodukt auf.

Selten findet man Golgi-Felder, die eine genau umgekehrte Markierung der einzelnen Zisternen zeigen. Hier liegt ein starker Niederschlag

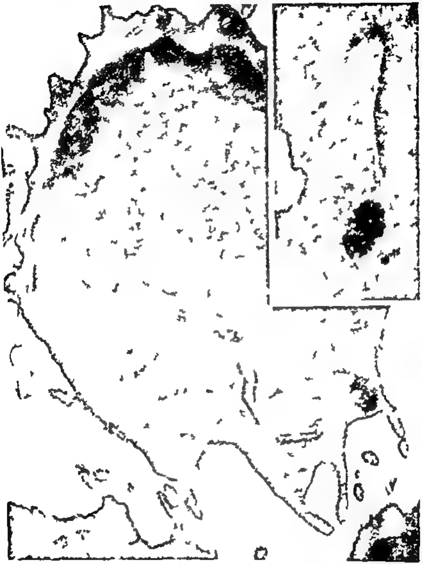


Abb 1 Kle ner Lymph zyt m t TPPase Reakt on in der au seren Lamelle des C lg Feldes. Das Pla malemm t negat v Z ka 30 000 Ausschn tt Lysosom m t pos t er Reakt on auf TPPase Z ka 40 000

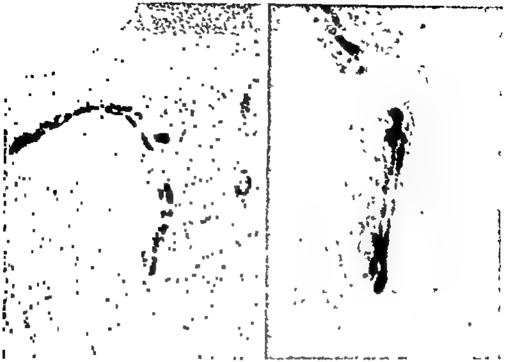
a  b

Abb 2. *a* TPPase-Aktivität in den konvexen Lamellen des Golgi-Feldes. Zirka × 50 000. *b* Kräftige TPPase-Reaktion in kolbenförmigen Endauftreibungen der Golgi-Lamellen. Zirka × 57 000

in den inneren konkaven Elementen (Abb. 3a, b). Verändert man den pH-Wert der Inkubationslösung, so ergibt sich ein deutlicher Abfall des Reaktionsproduktes im sauren Bereich; meistens ist der Nachweis negativ. Eine Inkubation ohne Substrat lässt ebenfalls keinen spezifischen Niederschlag entstehen. Schwache Präzipitatbildungen im Zytoplasma, *ohne dass eine Zuordnung zu bestimmten Zellstrukturen möglich ist, sind* als unspezifische Hydrolyseprodukte anzusehen. Sie treten in gleicher Verteilung auch nach Inkubation ohne Substrat auf. Tauscht man im Medium das Substrat TPP gegen UDP aus, so tritt bei pH 7,2 im Golgi-Feld ein Niederschlag auf, der aber sehr locker in den einzelnen Zisternen verteilt ist. Zusätzlich ist an der Zellmembran schwaches Reaktionsprodukt zu finden (Abb. 4). Die Reaktionsstärke an der Zellmembran nimmt mit fallendem pH zu und ist bei pH 5,5 am kräftigsten. In gleichem Masse wie die Aktivität an der Plasmamembran zunimmt, kommt es im Golgi-Feld zu einem negativen Ausfall der Reaktion, so dass bei pH 5,5 eine UDPase-Aktivität nur noch an der Zellmembran lokalisierbar ist (Abb. 5).

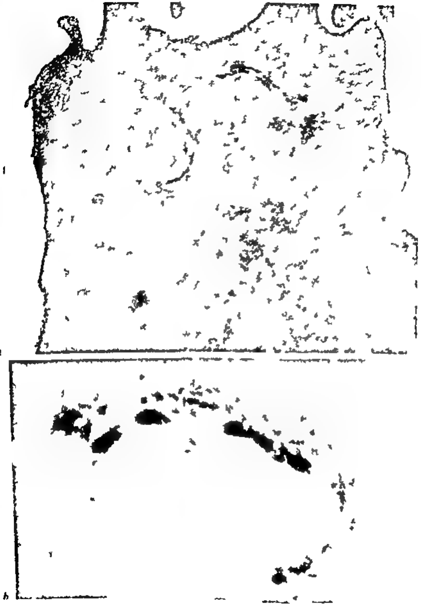


Abb. 3. TPPase-Aktivität in den ... konkaven Lamellen des Golgi-Feldes. *a* Z.-ka. 35 000; *b* Z.-ka. 90 000

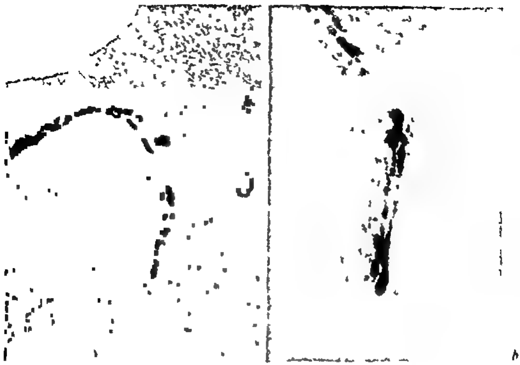


Abb 2 a TPPase Aktivität in den konvexen Lamellen des Golgi Feldes Zirka × 50 000 *b* Kräftige TPPase-Reaktion in kolbenförmigen Endauftreibungen der Golgi Lamellen Zirka × 57 000

in den inneren konkaven Elementen (Abb 3a, b) Verändert man den pH-Wert der Inkubationslösung, so ergibt sich ein deutlicher Abfall des Reaktionsproduktes im sauren Bereich, meistens ist der Nachweis negativ *Eine Inkubation ohne Substrat lässt ebenfalls keinen spezifischen* Niederschlag entstehen Schwache Präzipitatbildungen im Zytoplasma ohne dass eine Zuordnung zu bestimmten Zellstrukturen möglich ist, sind als unspezifische Hydrolyseprodukte anzusehen Sie treten in gleicher Verteilung auch nach Inkubation ohne Substrat auf Tauscht man im Medium das Substrat TPP gegen UDP aus so tritt bei pH 7,2 im Golgi Feld ein Niederschlag auf, der aber sehr locker in den einzelnen Zisternen verteilt ist Zusätzlich ist an der Zellmembran schwaches Reaktionsprodukt zu finden (Abb 4) Die Reaktionsstärke an der Zellmembran nimmt mit fallendem pH zu und ist bei pH 5 5 am kräftigsten In gleichem Masse wie die Aktivität an der Plasmamembran zunimmt, kommt es im Golgi Feld zu einem negativen Ausfall der Reaktion, so dass bei pH 5,5 eine UDPase Aktivität nur noch an der Zellmembran lokalisierbar ist (Abb 5)

Abb. 3 TPPase-Aktivität in den inneren konkaven Lamellen des Golgi-Feldes. *a* Zirka 35 000, *b* Zirka 90 000

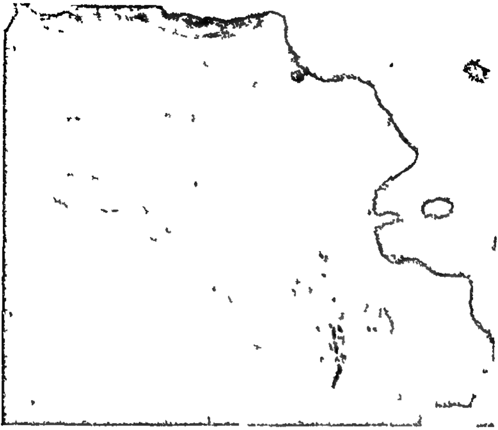


Abb 4 UDPase Aktivitat im Golgi Feld und an der Plasmamembran bei pH 7 2 Zirka × 36 000

Diskussion

Die DT Lymphozyten der Ratte setzen sich aus zirka 85% T Lymphozyten und 12–15% B Lymphozyten zusammen [29] Zieht man den Phanotyp der peripheren B Zelle und T-Zelle, wie sie im Scanningmikroskop von POLLIACK *et al* [27] beschrieben worden sind, zur Identifizierung der TPPase und NDPase positiven Lymphozyten heran so fallt auf, dass TPPase positive Lymphozyten im Schnittbild immer eine mit sehr vielen fingerformigen Fortsatzen versehene Zellmembran besitzen (Abb 1) Dies ist ein Merkmal fur B Lymphozyten [27] Demgegenuber sind NDPase positive Zellen uberwiegend kugelige Lymphozyten ohne starke Gliederung der Zellmembran (Abb 5) entsprechend den T-Zell-Merkmalen [20 21 27] Ob diese Befunde an definierten B bzw

Abb. 5 UDPase-Aktivität bei pH 5,5 ist nur an der Plasmamembran zu finden, während das Golgi-Feld (GO) frei bleibt. Zirka × 30000

T-Lymphozyten erhärtet werden können, wird zurzeit noch untersucht. Ihre Interpretation gestaltet sich aufgrund der Problematik, die ein histochemischer Nachweis von TPPase und NDPase mit sich bringt [2, 3, 13, 15, 23], sicher nicht zufriedenstellend, jedoch mögen einige spekulative Deutungen erlaubt sein.

Die histochemische Lokalisation der TPPase im Golgi-Feld vieler Säuger- und Pflanzenzellen macht dieses Enzym zu einem zytochemischen Marker des Golgi-Feldes [9]. Die hohe Aktivität der TPPase in stark sekretorisch tätigen Zellen konnte darauf hinweisen, dass Diphosphatasen allgemein entweder an der Ausschleusung oder an einigen Schritten der Synthese von Sekreten beteiligt sind. Bei der Bildung der Glykolipide und der Glykoproteine, die als Membranbausteine oder in der Glykokalyx Verwendung finden oder aber als Antikörper ausgeschleust werden, wird eine Beteiligung der Diphosphatasen diskutiert [14, 34]. Die Lymphozytenoberfläche ist eine sehr dynamische Membran, an der die Umsatzraten der einzelnen Bausteine im Durchschnitt wenige Stunden betragen [19]. Bei Milz-B-Lymphozyten waren innerhalb von 6 h mehr als 40% der Oberflächenimmunglobuline ausgetauscht [36]. MELCHERS und ANDERSSON [22] haben nachweisen können, dass sich in B-Lymphozyten zirka 90% des neugebildeten IgM nach 4 h noch in der Zelle befanden. Nach einer Verzögerung von 1 h begann die Ausschleusung, und nach weiteren 4 h war ebenfalls mehr als die Hälfte des IgM von der Lymphozytenmembran abgegeben.

Neben der Bereitstellung von Immunglobulinen als Oberflächenrezeptoren von B-Zellen steht die Produktion anderer Glykoproteine und Glykolipide für die Oberflächenschicht sicher an weiterer dominierender Stelle, zumal dieser Teil dem Lymphozyten seine wichtige biologische und immunkompetente Stellung verleiht [38]. Diese Produkte werden über Golgi-Vesikel an die Plasmamembran herangeführt und dann entweder in das umgebende Milieu entlassen oder in die Plasmamembran mit eingebaut [36]. Dieser Vorgang erfordert eine ständige Membranneubildung, und deswegen wird das Golgi-Feld von einer Reihe von Autoren auch als Ort des Phospholipidmetabolismus angesehen [8, 33]. TPP-hydrolysierende Enzyme sollen dabei die Menge des Acetyl-CoA beeinflussen, das in Membranphospholipide eingeschleust wird. Auch hierfür konnte die TPPase-Aktivität in B-Lymphozyten Ausdruck sein.

Die vorliegenden Befunde über Lokalisation von TPPase in Golgi-Feld und Lysosomen und von NDPase an der Zellmembran sprechen dafür, dass es sich hier um zwei verschiedene Enzyme handelt. Hierbei ist die pH-Abhängigkeit der NDPase von besonderem Interesse. Während bei neutralem pH im Golgi-Feld eine leichte NDPase-Aktivität auftritt, verschiebt sich der Reaktionsort im sauren Bereich zugunsten der Aktivität an der Plasmamembran. Nimmt man an, dass ein Enzym nur ein pH-Optimum besitzt, so könnte es sich bei der NDPase des Golgi-

Feldes und der des Plasmalemms um zwei verschiedene Enzyme handeln die beide NDPase spalten können Diese Vorstellung wird auch dadurch bekräftigt, dass unterschiedliche Funktionen etwa als Synthetasen und Transferasen angenommen werden [10] Eine weitere Erklärung könnte darin zu sehen sein, dass die Aktivität im Golgi Feld, als dem Produktionsort vieler Enzyme, eine Reaktion *in statu nascendi* ist die Hauptaktivität aber erst an der Zellmembran entfaltet wird

Die Bedeutung von Glykosyltransferasen für die Zelladhäsion und für das gegenseitige Erkennen von Zellen darf als gesichert gelten Hierbei spielen Nucleosidasen, vor allem UDPase, eine wichtige Rolle [16, 30, 31] Zwar stehen für den Nachweis der Glykosyltransferasen keine histochemischen Methoden zur Verfügung doch kann die Lokalisation von Nucleosidasen unter Umständen als indirekter Nachweis für Glykosyltransferasen aufgefasst werden Dass T-Lymphozyten eine deutliche NDPase-Aktivität an der Zellmembran aufweisen, erscheint sinnvoll, da gerade die T-Lymphozyten permanent mit der Diskriminierung von eigenen und nicht eigenen zellulären Antigenen befasst sind

Ähnlich wie die funktionelle Bedeutung der unspezifischen Esterase von T-Lymphozyten noch unklar ist [25] müssen für die NDPase weitere funktionelle Möglichkeiten untersucht werden z.B ihre Rolle bei der endozytotischen Aufnahme von Antigen oder bei dem zytotoxischen Effekt von aktivierten T-Zellen auf ihre Target Zellen

Zusammenfassung

An B- und T-Lymphozyten wird die elektronenmikroskopische Lokalisation von TPPase und UDPase untersucht Hierbei zeigt sich dass die TPPase im Golgi Feld und in Lysosomen lokalisiert werden kann während UDPase abhängig vom pH Wert im Golgi Feld und an der Plasmamembran gefunden werden kann Die funktionelle Bedeutung der nachgewiesenen Enzyme bei B und T Zellen wird diskutiert

Literatur

1 Ackermann A G Cytochemistry of the lymphocytes in Rebuck The lymphocyte and lymphatic tissue (Hoeber New York 1961)

2 Allen J M The properties of golgi associated nucleosidediphosphatase and thiamine pyrophosphatase I Cytochemical analyses J Histochem Cytochem *11* 529 541 (1963)

3 Allen J M The properties of golgi associated nucleosidediphosphatase II

Electrophoretic separation and identification J Histochem Cytochem *11* 542–552 (1963)

4 ANTENIUS A Origin and fate of multivesicular bodies in PHA stimulated lymphocytes Cell Tiss Res *149* 497–511 (1974)

5 ASTALDI G CICHOCKI T and LISIEWICZ J Lysosomal enzyme content in lymphocytes in TAKEUCHI Proc 4th Int Congr Histochemistry and Cytochemistry Kyoto 1972

6 BOLLMANN J L CAIN J C and GRINDLEY J H Techniques for collection of lymph from the liver small intestine or thoracic duct of the rat J Lab clin Med *33* 1349–1354 (1948)

7 BOWERS W E Lysosomes in rat thoracic duct lymphocytes J exp Med *136* 1394–1403 (1972)

8 COHN Z A and BENSON B The *in vitro* differentiation of mononuclear phagocytes I The influence of inhibitors and the results of autoradiography J Cell Biol *121* 279 288 (1965)

9 DAUWALDER M WHALEY W G and KEPHART J E Functional aspects of golgi apparatus Sub cell Biochem *1* 225–275 (1972)

10 ERNSTER L and JONES L C A study of nucleoside tri and di phosphate activities of rat liver microsomes J Cell Biol *15* 563–578 (1962)

11 FRIESS A E und LIEBICH H G Ultrahistochemischer Nachweis von Phosphatasen und Peroxidase in Zellen des Ductus thoracicus Acta histochem suppl 8 pp 195 202 (1973)

12 FRIESS A E Elektronenmikroskopische Enzym Substrat und immunchemische *Untersuchungen an Lymphozyten aus dem Ductus thoracicus der Ratte* Habschr Regensburg (1973)

13 GOLDFISCHER S ESSNER E and SCHILLER B Nucleoside diphosphatase and thiaminepyrophosphatase activities in the endoplasmatic reticulum and golgi apparatus J Histochem Cytochem *19* 349–360 (1971)

14 HEATH E C Complex polysaccharides Ann Rev Biochem *40* 29 56 (1971)

15 HÜNDGEN M Beitrag zur Klarung der Spezifität Thiaminpyrophosphat und Nucleosiddiphosphate spaltender Enzyme Histochemie *22* 376–382 (1970)

16 KEMP R B LLOYD C W and COOK G M W in DANIELLI ROSENBERG and CADENHEADS Progress in surface and membrane science Vol 7 pp 271 318 (Academic Press London 1973)

17 KOUVALAINEN K Species and organ dependence of alkaline phosphatase activity in lymphatic tissue A histochemical biochemical and electrophoretic study J Histochem *3* 55–69 (1971)

18 LENNERT K und LÖFFLER H Fermenthistochemische Untersuchungen der Antikorperbildungsstatten Verh dt Ges Path *46* 115 118 (1962)

19 LERNER R A MCCONAHEY P J and DIXON F J Synthesis of plasma membrane associated and secretory immunglobulin in diploid lymphocytes J exp Med *135* 136–149 (1972)

20 MATTER A LISOWSKA BERNSTEIN B RYSER J E LAMELIN J P and VASALLI P Mouse thymus independent and thymus derived lymphoid cells II Ultrastructure studies J exp Med *136* 1008–1018 (1972)

21 MATTER A The differentiation pathway of T lymphocytes J exp Med *140* 566–577 (1974)

22 MELCHERS, F. and ANDERSSON, J.: Synthesis, surface, deposition and secretion of IgM in bone marrow derived lymphocytes before and after mitogenic stimulation. Transplantn Rev. *14:* 76–130 (1973).
23 MELDOLESI, J.; JAMIESON, J. O., and PALLADE, G. E.: Composition of cellular membranes in the pancreas of the guinea pig. III. Enzymatic activities. J. Cell Biol. *49:* 150–158 (1971).
24 MÜLLER-HERMELINK, H. K.; HEUSERMANN, U., and STUTTE, H. J.: Enzyme histochemical observations on the localization and structure of the T-cell and B-cell regions in the human spleen. Cell Tiss. Res. *154:* 167–179 (1974).
25 MÜLLER, J.; BRUN DEL RE, G.; BÜRKI, H.; KELLER, H. U.; HESS, M. W., and COTTIER, H.: Nonspecific acid esterase activity: a criterion for differentiation of T- and B-lymphocytes in mouse lymph nodes. Eur. J. Immunol. *5:* 270–274 (1975).
26 NOVIKOFF, A. B. and GOLDFISCHER, S.: Nucleosidediphosphatase activity in the golgi apparatus and its usefulness for cytological studies. Proc. natn. Acad. Sci. USA *47:* 802–810 (1961).
27 POLLIACK, A.; LAMPEN, N.; CLARKSON, B. O., and HARVEN, E. DE: Identification of human B and T lymphocytes by scanning electron microscopy. J. exp. Med. *138:* 607–624 (1973).
28 QUASTEL, M. R. and KAPLAN, J. G.: Lymphocyte stimulation: the effect of Ouabain on nucleic acid and protein syntheses. Exp. Cell Res. *62:* 407–420 (1970).
29 RAFF, M. C.; STERNBERG, M., and TAYLOR, R. B.: Immunglobulin determinants on the surface of mouse lymphoid cells. Nature, Lond. *225:* 553–554 (1970).
30 ROSEMAN, S.: The synthesis of complex carbohydrate by multiglycosyltransferase system and their potential function in intercellular adhesion; in Chemistry and physics of lipids (North Holland, Amsterdam 1970).
31 ROTH, S.; MCGUIRE, E. J., and ROSEMAN, S.: Evidence for cell surface glycosyltransferase. Their potential role in cellular recognition. J. Cell Biol. *51:* 536–547 (1971).
32 RUSKAMEN, O. and KOUVALAINEN, K.: The fate of thymocytes. A study of kinetics of thymocytes using high alkaline phosphatase activity as an endogenous label. Scand. J. Haematol. *9:* 174–185 (1972).
33 SJÖSTRAND, F. S.: Fine structure of exocrine pancreas cells; in Ciba Foundation Symp. Exocrine Pancreas, pp. 1–19 (Little, Brown, Boston 1962).
34 SPIRO, R. G.: Glykoproteins. Ann. Rev. Biochem. *39:* 599–638 (1970).
35 TAMAOKI, N. and ESSNER, E.: Distribution of acid phosphatase, β-glucuronidase and N-acetyl-β-glucosaminidase activities in lymphocytes of lymphatic tissues of man and rodents. J. Histochem. Cytochem. *17:* 238–243 (1969).
36 VITETTA, E. S. and UHR, J. W.: Synthesis, transport, dynamics and fate of cell surface Ig and alloantigens in murine lymphocytes. Transplantn Rev. *14:* 50–75 (1973).
37 WHITNEY, R. B. and SUTHERLAND, R. M.: Enhanced uptake of calcium by transforming lymphocytes. Cell. Immunol. *5:* 137–147 (1972).
38 WIGZELL, H.: The functional significance of surface structures on lymphocytes. Annls Inst. Pasteur, Paris *124C:* 7–15 (1973).

Dr. A. F. FRIESS, Institut für Anatomie der Universität Regensburg, Universitätsstrasse 31, *D-84 Regensburg* (BRD)

Acta haemat. 55: 216–223 (1976)

Impairment of Platelet Adhesiveness and Platelet Factor 3 Activity in Cyanotic Congenital Heart Disease

M. Bhargava, S. K. Sanyal, M. K. Thapar, S. Kumar and V. Hooja

Department of Pathology, Lady Hardinge Medical College and Hospital, and Department of Paediatrics, Safdarjang Hospital, New Delhi

Key Words. Congenital heart disease · Haemostasis · Platelet adhesiveness · Platelet factor 3 · Platelet function · Polycythemia

Abstract. In 33 children with cyanotic congenital heart disease the platelet function has been studied. The most significant changes were reduced platelet adhesiveness to glass and impaired availability of platelet factor 3 in nearly 50% of the patients. Although clot retraction was poor in 84% of them, thrombocytopenia and prolonged bleeding time were not significant features.

Bleeding diathesis in cyanotic congenital heart disease (CCHD), leading to severe and even fatal postoperative haemorrhage has been recognised for some time. A wide variety of haemostatic abnormalities have been reported to be present preoperatively. Thrombocytopenia [12, 20, 24], defective clot retraction [1, 7, 16], prolonged prothrombin time [15, 16, 18, 24], prolonged partial thromboplastin generation time [1, 5, 17], hypofibrinogenaemia and accelerated fibrinolysis [12, 20] have been noted. Recently, defective aggregation of platelets to ADP or collagen has been recognised [11, 13, 22]. To our knowledge, in none of the available studies impairment of platelet adhesion or platelet factor 3 activity has been found. The purpose of this communication is to describe these platelet abnormalities in CCHD. Coagulation defects were excluded by appropriate laboratory tests.

Fig 1 Apparatus for measuring platelet adhesiveness to glass

Materials and Methods

33 patients of both sexes were evaluated Their ages varied from 1 to 12 years The diagnoses included Fallot s tetralogy, total anomalous pulmonary venous drainage, tricuspid atresia and transposition of great vessels None of the patients displayed evidence of abnormal bleeding at the time of study They had not received aspirin or any other drug known to alter platelet function for at least 10 days prior to study None of the patients had evidence of renal or liver disease

For tests of coagulation and platelet function venous blood was collected in siliconised syringes and 9 vol added to 1 vol of 3 2% trisodium citrate The amount of citrate was adjusted proportionally to the volume of packed red cells (VPRC) in polycythaemic subjects with VPRC above 45%, using the formula

$$x = 100 - VPRC\ (\text{patient})\ 55$$

where x = volume of citrate in ml to which whole blood is added to a final volume of 10 ml Each patient was studied with an age matched control

The methodologic difficulties involved in separating platelet rich plasma (PRP) to study platelet function in thrombocytopenic children with polycythaemia are formidable Insufficient quantities of PRP obtained resulted in an unequal number of tests in some instances

Haemoglobin was determined as cyanmethaemoglobin using Drabkin s solution VPRC was determined by Wintrobe s method Routine platelet counts were performed with ammonium oxalate as the diluent according to HARDISTY and INGRAM [9] Bleeding time was measured by Ivy s method [4] and clot retraction according to BIGGS and MACFARLANE [2]

Platelet adhesiveness to glass was tested *in vitro* by a modified Salzman s technique The apparatus was assembled at the National Physical Laboratory Delhi (fig 1) Blood from a clean venipuncture was allowed to flow through a glass column 9 cm long containing 2.5 g of glass beads of 0 5 mm diameter each at a constant pressure of 100 mm Hg for a period of 20 sec in each instance Platelet aggregation to ADP was measured according to HARDISTY and INGRAM [9] and to collagen by the method of ZUCKER and BORRELLI [26] Platelet factor 3 (Pf 3) availability was determined by the method based on kaolin clotting time of dilutions of PRP as described by HARDISTY and HUTTON [10]

Acta haemat. 55: 216–223 (1976)

Impairment of Platelet Adhesiveness and Platelet Factor 3 Activity in Cyanotic Congenital Heart Disease

M. BHARGAVA, S. K. SANYAL, M. K. THAPAR, S. KUMAR and V. HOOJA

Department of Pathology, Lady Hardinge Medical College and Hospital, and Department of Paediatrics, Safdarjang Hospital, New Delhi

Key Words. Congenital heart disease · Haemostasis · Platelet adhesiveness · Platelet factor 3 · Platelet function · Polycythemia

Abstract. In 33 children with cyanotic congenital heart disease the platelet function has been studied. The most significant changes were reduced platelet adhesiveness to glass and impaired availability of platelet factor 3 in nearly 50% of the patients. Although clot retraction was poor in 84% of them, thrombocytopenia and prolonged bleeding time were not significant features.

Bleeding diathesis in cyanotic congenital heart disease (CCHD), leading to severe and even fatal postoperative haemorrhage has been recognised for some time. A wide variety of haemostatic abnormalities have been reported to be present preoperatively. Thrombocytopenia [12, 20, 24], defective clot retraction [1, 7, 16], prolonged prothrombin time [15, 16, 18, 24], prolonged partial thromboplastin generation time [1, 5, 17], hypofibrinogenaemia and accelerated fibrinolysis [12, 20] have been noted. Recently, defective aggregation of platelets to ADP or collagen has been recognised [11, 13, 22]. To our knowledge, in none of the available studies impairment of platelet adhesion or platelet factor 3 activity has been found. The purpose of this communication is to describe these platelet abnormalities in CCHD. Coagulation defects were excluded by appropriate laboratory tests.

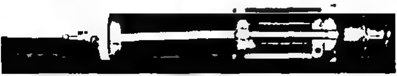


Fig. 1. Apparatus for measuring platelet adhesiveness to glass.

Materials and Methods

33 patients of both sexes were evaluated. Their ages varied from 1 to 12 years. The diagnoses included Fallot's tetralogy, total anomalous pulmonary venous drainage, tricuspid atresia and transposition of great vessels. None of the patients displayed evidence of abnormal bleeding at the time of study. They had not received aspirin, or any other drug known to alter platelet function, for at least 10 days prior to study. None of the patients had evidence of renal or liver disease.

For tests of coagulation and platelet function, venous blood was collected in siliconised syringes and 9 vol added to 1 vol of 3.2% trisodium citrate. The amount of citrate was adjusted proportionally to the volume of packed red cells (VPRC) in polycythaemic subjects with VPRC above 45%, using the formula

x = 100 − VPRC (patient)/55

where x = volume of citrate in ml to which whole blood is added to a final volume of 10 ml. Each patient was studied with an age matched control.

The methodologic difficulties involved in separating platelet rich plasma (PRP) to study platelet function in thrombocytopenic children with polycythaemia are formidable. Insufficient quantities of PRP obtained resulted in an unequal number of tests in some instances.

Haemoglobin was determined as cyanmethaemoglobin using Drabkin's solution. VPRC was determined by Wintrobe's method. Routine platelet counts were performed with ammonium oxalate as the diluent according to HARDISTY and INGRAM [9]. Bleeding time was measured by Ivy's method [4] and clot retraction according to BIGGS and MACFARLANE [2].

Platelet adhesiveness to glass was tested *in vitro* by a modified Salzman's technique. The apparatus was assembled at the National Physical Laboratory, Delhi (fig. 1). Blood from a clean venipuncture was allowed to flow through a glass column 9 cm long, containing 2.5 g of glass beads of 0.5 mm diameter each, at a constant pressure of 100 mm Hg for a period of 20 sec in each instance. Platelet aggregation to ADP was measured according to HARDISTY and INGRAM [9] and to collagen by the method of ZUCKER and BORELLI [26]. Platelet factor 3 (Pf 3) availability was determined by the method based on kaolin clotting time of dilutions of PRP as described by HARDISTY and HUTTON [10].

Table I Frequency of platelet defects in cyanotic congenital heart disease

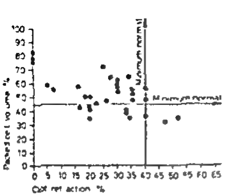
Test	Number of patients investigated	Patients showing the defect	
		number	%
Platelet count	30	5	16.6
Bleeding time	28	3	10.7
Clot retraction	32	27	84.3
Platelet adhesiveness to glass	28	13	46.4
Platelet aggregation to			
ADP	27	0	[illegible]
Collagen	11	5	46.0
Pf 3 activity	24	12	50.0

Table II Severity of platelet defects in cyanotic congenital heart disease (mean ± SD)

Test	Patients	Controls	p	t
Platelet count $\times 10^3/\mu l$	232.0 ± 86.0	368.9 ± 84.7		
Bleeding time min	3.7 ± 2.0	2.3 ± 1.3		
Clot retraction %	25.7 ± 12.8	44.7 ± 5.8	<0.0005	2.64
Platelet adhesiveness %	30.0 ± 15.9	39.0 ± 4.[illegible]	<0.01	2.64
Platelet aggregation with				
ADP sec	10.2 ± 5.0	8.8 ± 4.6	>0.05	0.828
Collagen %	35.0 ± 19.0	39.0 ± 8.5	>0.05	0.64
Pf 3 availability, %	4.7	>25		

Results

In the 33 patients studied, haemoglobin below 9 g% was seen only in 9, in the remaining it ranged between 13 and 22 g%. 17 children showed VPRC of 50% and above. The incidence of abnormal haemostasis was 100%. In 25 of the 33 children, two or more haemostatic abnormalities were detected. Platelet dysfunction was isolated in 11 patients while in 18 it coexisted with coagulation defects. The abnormal haemostatic profile was characterised by impairment of clot retraction, reduced platelet adhesion to glass and poor availability of Pf-3 (table I). As many as 27 of the 33 patients investigated showed poor syneresis of the clot. Amongst these

2 

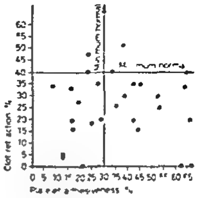
 3

Fig 2 Relation of packed cell volume to clot retraction
Fig 3 Relationship between clot retraction and platelet adhesiveness.

27, 4 were thrombocytopenic and 14 had VPRC above 50%. Nearly half of the patients showed impaired platelet adhesiveness and aggregation to collagen. Almost an equal number exhibited poor availability of Pf-3. In none was the platelet aggregation to ADP defective. Thrombocytopenia or prolonged bleeding time were not significant features, being present only in 3 and 5, respectively, of the 28 and 30 patients investigated.

The severity of the platelet defects in cyanotic children is summarised in table II. Analysed statistically, the significant abnormalities comprised impaired clot retraction, reduced platelet adhesion to glass, and poor availability of Pf-3. Platelet count, bleeding time and aggregation of platelets to ADP were not different from those of controls. Likewise, an apparent decrease in platelet aggregation to collagen was statistically not significant.

Since anoxic polycythaemia of varying degree is a common denominator to all patients with cyanotic variety of congenital heart disease, the haemostatic defects were correlated with VPRC. Patients who suffered from multiple haemostatic defects had significantly higher median VPRC than those with a single defect ($p<0.05$, $t = 1.84$). While none amongst the latter had VPRC greater than 60%, it was the case at least in 7 children who had several haemostatic defects.

In general, the higher the VPRC, the poorer was the clot retraction. A correlative study between the two (fig. 2) revealed that of the 27 children

who showed impaired retraction of the clot, 19 had VPRC more than 45% Of the latter, in every instance the clot retraction was poor

No relationship, however, existed between platelet adhesion to glass and the VPRC In 19 patients with VPRC above 45%, adhesiveness of platelets to glass was impaired in 10 and normal in 9 In fact, in 10 of the 13 children it was decreased in spite of the raised VPRC On the other hand, at least in some of the patients, platelet adhesiveness seemed to influence the clot retraction Thus, in 13 children the platelet adhesion was subnormal, and in all except two the clot retraction was also poor (fig 3) However, an equal number of patients did not show any impairment in platelet adhesiveness although their clot retraction was decreased

Discussion

Our results demonstrate a high incidence of haemostatic defects in children suffering from CCHD The study further brings out the role of platelet dysfunction in postoperative bleeding so frequently observed in this disorder A defect of platelet function was demonstrable in nearly 60% of the cases In one third, it constituted the sole abnormality Impaired adhesion of platelets to glass and decreased availability of Pf-3 were characteristic changes We are not aware of any study describing these changes in CCHD The only platelet dysfunction recognised so far is defective aggregation to ADP or collagen

HELLEM *et al* [14] observed a direct correlation between the platelet adhesiveness and the VPRC We failed to find such a correlation On the contrary, in half of the patients showing high VPRC, the platelet adherence was reduced The reason for this is not clear A concomitant decrease in availability of Pf-3 in these children suggests that there may be a platelet membrane defect, inhibiting platelet reaction to greater concentrations of ADP released from a larger red cell mass

A number of physiological and biochemical changes in aging platelets have been reported in the past Old platelets as compared to young were less adhesive to glass [23, 25] and to collagen [15] Two independent studies indicate that old platelets were less effective in clot retraction [6, 21] Recently, JOHNSON *et al* [17] found aged platelets to be associated with long bleeding times, decreased platelet adhesiveness *in vivo* and *in vitro* as well as poor availability of Pf-3 Thus, it would be not unreasonable to assume that a premature aging of platelets brought about by a per-

sistent anoxic state in CCHD patients may be responsible for the abnormalities of platelet function found in these children

A normal aggregation of platelets to ADP was not accompanied by normal release of Pf-3, as could have been expected This divergence can best be explained by the fact that in the Pf-3 test used, kaolin contact and not aggregation was the agent triggering release of platelet contents However, there is evidence to suggest that total phospholipid content of aging platelets may indeed be less [8, 19]

Poor clot retraction chractensed 84% of the cases It is well known that changes in the platelets or increase in the red cell mass significantly impair the ability of the clot to retract [3] Considering that these children showed negligible thrombocytopenia, the defective clot retraction would appear to be due to an increase in the red cell mass Raised VPRC in 70% of the children showing impaired clot retraction would distinctly seem in favour this possibility

Eleven of the 13 children showing poor clot retraction also exhibited abnormal platelet adhesiveness This suggests that poor platelet adhesion may be an additional factor causing impairment of clot retraction If so, its mechanism may be related to defective release of thrombosthenin from a decrease in platelet adhesion

Since none of the children showed bleeding preoperatively, it would appear that an altered platelet function together with deficient coagulation factor(s) may result in a basically incompetent haemostatic status which fails to maintain an equilibrium postoperatively Appropriate screening tests performed preoperatively can detect the bleeding diathesis and thereby help in its management

Acknowledgements This study was supported by a grant from the Indian Council of Medical Research The authors are grateful to Mr A P Chaturvedi for technical assistance

References

1 Atachlas [illegible] Hein De Balsac R Guéry J Passelecq J Bloudeau P et Dubost C Les thrombopathies associées aux cardiopathies congénitales étude de l'hémostase dans cinquante cas Revue fr Clin biol 3 322 (1958)

2 Biggs R and Macfarlane R G Human blood coagulation and its disorders (Blackwell Oxford 1962)

3 Cohn M Jackson D P, and Conley C L Components of blood necessary for clot retraction Bull Johns Hopkins Hosp 107 90 104 (1960)

4 Dacie, J V and Lewis S M Practical haematology (Churchill, London 1968)

5 DENNIS L H STEWART J L and CONRAD M E Heparin treatment of haemorrhagic diathesis in cyanotic congenital heart disease Lancet i 1088–1089 (1967)

6 DETWILER J C ODELL T T jr and MC DONOLD T P Platelet size ATP contents and clot retraction in relation to platelet age Am J Physiol *203* 107–113 (1962)

7 FAVRE GILLY J BRET J et BORLL MILHET J Un trouble inattendu de la coagulation dans la maladie bleue l hypoprothrombinémie son importance en clinique et dans le problème de l hémostase au cours des opérations de Blalok et de Potts Sang *22* 278 287 (1951)

8 GINSBURG A J and ASTER R H Changes associated with platelet aging Atlantic City Meetings p 325 (American Federation of Clinical Research 1969)

9 HARDISTY R M and INGRAM G I C Bleeding disorders investigation and management (Blackwell Oxford 1965)

10 HARDISTY R M and HUTTON R A The kaolin clotting time of platelet rich plasma a test of platelet factor 3 availability Br J Haemat *11* 258 268 (1965)

11 HARDISTY R M and HUTTON R A Bleeding tendency associated with new abnormality of platelet behaviour Lancet i 983 985 (1967)

12 HARTMAN R C A haemorrhagic disorder occurring in patients with cyanotic congenital heart disease Bull Johns Hopkins Hosp *91* 49 67 (1952)

13 HATHAWAY, W E Bleeding disorders due to platelet dysfunction Am J Dis Child *121* 127 134 (1971)

14 HELLEM A J BORCHGREVINCK C F and AMES S B Role of red cells in haemostasis relation between haematocrit bleeding time and platelet adhesiveness Br J Haemat 7 42 50 (1961)

15 HIRSH J GLYNN M F and MUSTARD J F The effect of platelet age on platelet adherence to collagen J clin Invest *47* 466–473 (1969)

16 JACKSON D P Haemorrhagic diathesis in patients with cyanotic congenital heart disease Preoperative management Ann N Y Acad Sci *115* 235 251 (1964)

17 JOHNSON C A ABILDGAARD C F and SHULMAN I Absence of coagulation abnormalities in children with cyanotic congenital heart disease Lancet ii 660–662 (1968)

18 JOHNSON C A ABILDGAARD C F and SHULMAN I Functional studies of young versus old platelets in a patient with chronic thrombocytopenia Blood *37* 163–171 (1971)

19 KARPATKIN S Heterogeneity of human platelets I Metabolic and kinetic evidence suggestive of young and old platelets J clin Invest *48* 1073–1082 (1969)

20 KONTRAS S B SIRAK H D and NEWTON W A jr Haematologic abnormalities in children with congenital heart disease J amer med Ass *195* 611 (15 (1966)

21 LOHR G W and WALLER H D Zellstoffwechsel und Zellalterung (Cellular metabolism and cellular ageing) Klin Wschr *37* 833 (1969)

22 MAURER H M MCCUE C M CAUL J and STILL W J S Impairment in platelet aggregation in congenital heart disease Blood *40* 207–216 (1972)

23 Shulman, N R, Watkins, S B J, Itsocoitz, S B, and Students, A B Evidence that the spleen retains the youngest and haemostatically most effective platelets Trans Ass Am Physns *81* 302–313 (1968)
24 Sommerville, J, McDonald, L, and Edgill, M Postoperative haemorrhage and related abnormalities of blood coagulation in cyanotic congenital heart disease Br Heart J *27* 140–448 (1965)
25 Wright, H P Changes in the adhesiveness of blood platelets following parturition and surgical operations J Path Bact *54* 461–468 (1942)
26 Zucker M B and Borelli J Platelets clumping produced by connective tissue suspensions and by collagen Proc Soc exp Biol Med *109* 779–787 (1962)

Prof Manorama Bhargava Division of Haematology Department of Pathology Lady Hardinge Medical College New *Delhi – 110001* (India)

Acta haemat 55 224–229 (1976)

Growth of Haematopoietic Cells of Mouse Fetal Liver in Diffusion Chambers

J A Vilpo and Leena Vilpo

Department of Haematology, Turku University Hospital, and Department of Medical Microbiology, University of Turku Turku

Key Words Culture of haematopoietic cells · Diffusion chamber · Erythroblasts · Fetal haematopoiesis · Granulocytes · Macrophages

Abstract Growth of haematopoietic cells from fetal mouse liver of 14- to 16-day gestation was studied with the diffusion chamber technique The culture period varied from 1 to 8 days Cell proliferation and haemoglobin synthesis were seen in erythroblasts during the first days of culture However, the nearly pure erythroblastic population of the primary inoculum changed to vigorously growing granulocytic cells and macrophages during the 8 day assay period It seems likely that end product inhibition is operative within the monocyte macrophage and granulocyte cell lineages respectively, when cells grow in diffusion chambers *in vivo*

It has been shown that intraperitoneal diffusion chambers provide a good assay system for the study of normal and malignant haematopoiesis [2, 7, 12, 14, 16] The diffusion chamber technique seems to be particularly well suited for the quantitative evaluation of growth of haematopoietic cells [1] Murine haematopoietic stem cells proliferate and differentiate well in these closed *in vivo* cultures, granulocytes and macrophages usually dominate the cultures, whereas the occurrence of other cell lineages is more or less occasional [for a review, see Benestad and Breivik, 3]

It has been concluded that the diffusion chamber milieu does not facilitate the survival of bone marrow erythroid cells [1] Since fetal erythroid cells probably have a better growth potential, we chose liver haematopoietic cells of 14- to 16-day-old mouse fetuses for proliferation studies in diffusion chambers We here report how the initially nearly pure erythroblastic population changes to vigorously growing granulocytic and macrophagic cells during the 8-day assay period

Material and Methods

Mice of a random bred NMRI strain were used throughout the study. Diffusion chamber recipients were 6- to 10-week-old virgin females. Fetal mice of 14- to 16-day gestation served as cell donors. In two of the three culture series the chamber recipient mice were given 700 r of whole body irradiation 1 day prior to chamber implantation. The irradiation was produced by a 250 kV X ray apparatus. The physical conditions were: current 10 mA, 1.5 mm Cu filter, target distance 50 cm, and dose rate 85 r/min. The irradiation was omitted in the third culture series because many of the young mice did not survive the whole 8-day assay period after this treatment.

Fetal livers were removed surgically, cut into 2–3 mm pieces, and aspirated through progressively smaller needles (1.4–1.0–0.8–0.6–0.5 mm ∅). This resulted in a single cell suspension. The suspension medium was RPMI 1640 (Orion Pharmaceutical, Helsinki), containing 10% heat inactivated fetal calf serum (Flow Laboratories, Rockville, Md.). In the primary cell suspension about 95% of the nucleated cells were erythroblasts, and 2–4% granulocytic cells. Additionally, there were macrophages/monocytes, megakaryocytes, blasts, and some unidentified cells. A few liver parenchymal cells were also present.

Diffusion chambers with a capacity of about 130 μl were prepared of acrylic rings and Millipore filters as described earlier [15]. The chambers were filled with 100 μl of single cell suspension containing 5×10^6 nucleated cells. Two chambers glued together were implanted into the abdominal cavity of each recipient mouse. The mice were killed by neck luxation after 1, 2, 3, 5, and 8 days of culture. The chambers were cleaned with lens paper and then treated unopened in 0.5% Pronase solution (Pronase, B grade, Calbiochem, Los Angeles, Calif.) for 70 min. Cells were aspirated from the chambers and the inside of the chambers was washed five times with saline.

Cell counts were made haemocytometrically using acetic acid dilution to a final concentration of 1%. At least 100 nucleated cells were counted. Differential counts were made from cytocentrifuge preparations (Shandon Scientific, London) after staining with the May Grünwald-Giemsa technique. About 500 cells were counted for the differentials. Haematopoietic cells were classified in the following categories: (1) nucleated erythroid cells, (2) granulocytic cells, (3) macrophages (monocytes included), and (4) others.

Results

In cell numbers between cultures carried by pre-irradiated and untreated mice, statistically significant differences could not be demonstrated. Hence, results from the three culture series were combined. The total cell number did not change much during the whole 8-day assay period (fig. 1). The proportion of cells classified as 'others' (mainly blasts, but also megakaryocytes etc.) never exceeded 5% of the total harvest. Considerable differences were seen in the survival and growth of different cell types.

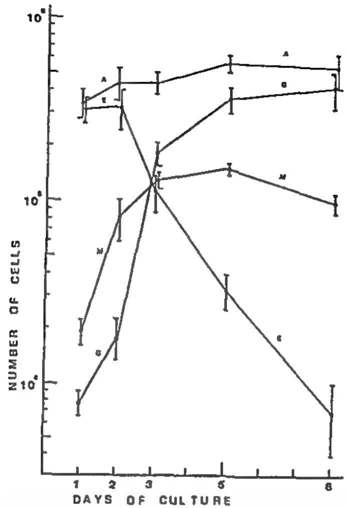


Fig 1 Growth of haematopoietic cells of mouse fetal liver in diffusion chambers Culture periods were 1, 2, 3, 5, and 8 days Primary inoculum was 5×10^5 nucleated cells. Values are mean (±SE) of 10 chambers (of 8 chambers on day 8) A = All cells; E = erythroblasts; G = granulocytic cells, M = macrophages (monocytes included)

The number of erythroblasts decreased with time (fig. 1). However, mitotic figures were seen in erythroid cells even on the third day of culture. The haemoglobin content of some erythroblasts increased during the first 2 or 3 days. It is also possible that nuclear ejection of normoblasts took place in the cultures; there were numerous small nuclei free and also phagocytized in the cytoplasm of macrophages. The number of granulocy-

tic cells increased strongly between days 1 and 3 (fig 1) The population doubling time for all granulocytic cells was about 10 h between days 1 and 3, and even shorter between days 2 and 3, being about 7 h In the beginning the majority of granulocytes were young proliferating cells, but the degree of maturation increased considerably during the culture The growth rate of granulocytic cells decreased after 3 days The net growth was nearly zero between days 5 and 8 From days 1 to 8 the total number of granulocytic cells had increased about fiftyfold. The number of cells classified as macrophages increased rapidly between days 1 and 3 (fig 1) Thereafter, the growth rate slowed down. After 5 days of culture the net growth became negative However, mitotic figures were still seen The percentage of other haematopoietic cells (group 4) did not markedly alter with time

Discussion

In the present work a new target cell population, namely fetal liver erythroblasts, was cultured It was not possible to identify all cell types reliably in the chamber cultures, in our opinion, classification of cells is indeed impossible on morphological grounds only However, this does not seriously invalidate the differential counts of the present work, because the great majority of cells (95% or more) belonged to the erythroid granulocytic, and macrophage series, which generally are easy to recognize Although the number of erythroid cells decreased during the culture, active erythropoiesis could be demonstrated during the first 3 days Hence, it is possible that the small positive net growth seen in the growth curve of erythroid cells between days 1 and 2 is real In addition to cell proliferation, haemoglobin synthesis was apparent. Despite an overall negative net growth of erythroid cells in chamber cultures fetal liver cell populations seem to be suitable for studying haemoglobin synthesis as well as erythropoiesis In fact, we have shown that subcutaneous erythropoietin injections into chamber bearing mice markedly increase the incorporation of ^{59}Fe into chamber cells although the basal rate of ^{59}Fe uptake is already high [unpublished results] The rapid proliferation of granulocytic cells ($T_{d/2}$~7 h) observed in this work is not unique, similar results have been presented earlier [6] The growth rate of granulocytic cells slowed down after 3 days of culture At the same time the majority of granulocytic cells began to be more mature, post mitotic cells, degenerated cell forms were also common in the cultures of longest duration.

The following conclusions concerning the control of granulopoiesis in the present assay system seem to be justified

(1) Granulopoiesis was not inhibited at the time when the number and the rate of loss of erythroid cells was highest In other words, erythroid cells or their degradation products do not affect granulopoiesis in a non-specific manner

(2) LAERUM *et al* [9] have shown that diffusion conditions through the membranes of the chambers are good for about 3–5 days Furthermore, the exponential growth of many kinds of cell lineages usually takes place in diffusion chamber cultures during the first week Hence, it seems likely that the decreased growth rate of granulocytic cells after 3 days as seen here was not caused by impaired nutritional conditions or accumulation of toxic substances inside the chambers

(3) It has been speculated [13] that an end product stimulation may play a role in the specific regulation of granulopoiesis In the present system a considerable part of granulocytic cells in older cultures were mature and partially degenerated However, any stimulation of granulopoiesis could not be detected

(4) The colony-stimulating factor, evidently produced by monocytes-macrophages [8, 11], may have a central role in the steering of granulopoiesis In the present culture system, the number of cells of the monocyte macrophage series was high between days 3 and 5, but no stimulation in the growth of the granulocytic cells could be noted Thus, a possibly colony stimulating factor originating inside the diffusion chambers does not seem to play a role in the steering of granulopoiesis

(5) Injection of the granulocyte chalone into mice carrying diffusion chambers inhibits DNA synthesis in proliferating granulocytes in the chamber cultures [4, 10] The inhibition of cell growth after 3 days of culture in the present system may also be explicable in terms of a feedback inhibition inside the granulocyte system Of course, the loss of granulocytes increases with time because there are more mature cells in older cultures

End product inhibition might also be operative within the monocyte-macrophage cell lineage when the net growth diminished since day 3 This has also been suggested by others [5, 6]

Acknowledgements The authors thank Docent TAPIO RYTÖMAA for his useful comments This study was financially supported by the Paulo Foundation Helsinki and the Emil Aaltonen Foundation Tampere

References

1 Benestad, H Formation of granulocytes and macrophages in diffusion chamber cultures of mouse blood leucocytes Scand J Haematol 7 279–288 (1970)

2 Benestad, H Cell kinetics in diffusion chambers Survival, resumption of proliferation and maturation rate of murine haemopoietic cells Cell Tiss. Kinet. 5 421–431 (1972)

3 Benestad, H and Breivik, H Murine haematopoiesis studied with the diffusion chamber technique Norwegian Defence Research Establishment Report No 61 (1972)

4 Benestad H, Rytömaa, T., and Kiviniemi, K. The cell specific effect of the granulocyte chalone demonstrated with the diffusion chamber technique Cell Tiss Kinet. 6 147–154 (1973)

5 Breivik, H and Benestad, H Regulation of granulocyte and macrophage formation in diffusion chamber cultures of mouse haematopoietic cells. Expl Cell Res 70 340–348 (1972)

6 Bøyum, A and Breivik, H Kinetics of murine haemopoietic cell proliferation in diffusion chambers Cell Tiss. Kinet. 6 101–110 (1973)

7 Bøyum A, Carsten, A, Laerum O., and Cronkite, E. Kinetics of cell proliferation of murine bone marrow cells cultured in diffusion chambers. Effect of hypoxia, bleeding, erythropoietin injections, polycythemia, and irradiation of the host. Blood 40 174–188 (1972)

8 Golde, D, Finley, T., and Cline, M Production of colony-stimulating factor by human macrophages. Lancet II 1397–1399 (1972)

9 Laerum O, Grünteisen, A., and Rajewsky, M Proliferative properties of malignant cell populations cultured in intraperitoneal diffusion chambers. Eur J Cancer 9 533–541 (1973)

10 Laerum O and Maurer, H Proliferation kinetics of myelopoietic cells and macrophages in diffusion chambers after treatment with granulocyte extracts (chalone) Virchows Arch Zellpath 14 293–305 (1973)

11 Moore, M and Williams, N Physical separation of colony stimulating cells from in vitro colony forming cells in haemopoietic tissue J cell comp Physiol 80 195–206 (1972)

12 Quesenberry, P, Niskanen, E Symann, M, Howard, D, Ryan, M, Halpern J., and Stohlman F., jr Growth of stem cell concentrates in diffusion chambers (DC) Cell Tiss. Kinet. 7 337–348 (1974)

13 Robinson W and Mangalik, A The kinetics and regulation of granulopoiesis. Seminars Hematol 12 7–25 (1975)

14 Squires, D The growth of human bone marrow in diffusion chambers. Br J Haemat 29 89–97 (1975)

15 Vilpo, J A Proliferation survival, and malignancy of chloroleukaemia cells grown in diffusion chambers in vivo Annls Med exp Biol Fenn. 50 165–174 (1972)

16 Vilpo, J A Differentiation of leukaemic cells in diffusion chambers. Scand J Haematol 10 367–377 (1973)

Dr J A Vilpo, Department of Haematology, Turku University Hospital, Kiinamyllynkatu 4–8, 20520 Turku 52 (Finland)

Acta haemat 55 230-233 (1976)

The Fibrinolytic Enzyme System in Anorexia Nervosa

D OGSTON and W.D OGSTON

Departments of Medicine and Mental Health, University of Aberdeen, Foresterhill, Aberdeen

Key Words Anorexia nervosa Fibrinogen Fibrinolysis Plasminogen activation

Abstract The levels of components of the fibrinolytic enzyme system in 7 patients with anorexia nervosa were compared with those of healthy young women Plasminogen activator levels were significantly higher and the mean fibrinogen concentration was lower in the anorexia nervosa patients

A number of studies have demonstrated that plasma fibrinolytic activity is impaired in obese persons [5, 13, 16] It has been reported that an increase in fibrinolytic activity occurs during weight reduction in the obese [4, 13], and that short term negative calorie balance also causes the fibrinolytic activity to increase in persons of normal body build [13] We report a study on the fibrinolytic enzyme system in patients with anorexia nervosa to determine whether chronic negative calorie balance is associated with a sustained increase in fibrinolytic activity and whether it results in alterations in the other components of the fibrinolytic system

Methods

Plasminogen activator was assayed by performing euglobulin clot lysis times by the method of NILSSON and OLOW [11] using a clot lysis time recorder (Carmanan Instrumentation, Glasgow) The results were expressed by plotting the times logarithmically against units of fibrinolytic activity [17] 10 U being arbitrarily equated with a lysis time of 50 min

Plasminogen was measured by the method of ALKJAERSIG *et al* [1] and expressed in Sherry units *Fibrin degradation products* (FR antigen FDP) *in serum* were assayed by the tanned red cell haemagglutination inhibition technique of MERSKEY *et al* [10]

Table I Components of the fibrinolytic enzyme in control subjects and patients with anorexia nervosa

Subject No	Age years	Height cm	Weight kg	O/S ratio	Plasminogen activator U	Plasminogen (casein) U/ml	Fibrinogen mg/dl	FDP µg/ml	α_1-Antitrypsin mg/dl	α_2-Macroglobulin mg/dl
Controls[1] *(n = 10)*										
	20 9 ±0 6	163 1 ±5 5	58.5 ±6 1	106 ±10	2.7 ±1 9	3 7 ±0 5	365 ±42	1 7 ±1 4	349 ±99	298 ±98
Anorexia nervosa patients										
1	19	167 6	38 1	66	7 8	4.2	300	0 3	–	–
2	20	160 0	35 0	65	7 2	4 3	310	5 1	300	376
3	[illegible]	167 6	39 0	68	4 7	4 6	300	[illegible]	368	312
4	16	166 4	43 1	75	5 0	3 9	300	2.9	255	254
5	35	157 5	31 8	62	6 0	3 4	–	–	–	–
6	17	162.6	31 8	58	9.2	3.2	275	3 8	360	396
7	26	179 1	40 4	63	7 0	3.5	250	1 2	330	300
Mean	23 7	165 8	37 0	65.3	6 7	3 9	289	3 4	323	328

[1] Mean ± SD

Fibrinogen was measured by a modification [12] of the method of RATNOFF and MENZIE [14]. *α_2-Macroglobulin and α_1-antitrypsin* levels were assayed by radial immunodiffusion [8] using reagents obtained from Behringwerke AG

Patients Seven patients with anorexia nervosa were studied. All were female and had lost weight through active refusal to eat. Amenorrhoea had been present at least 6 months, and in no patient were there features of schizophrenia, severe depression or organic disease. There was no evidence of hepatic dysfunction based on normal values of aspartate transaminase, alkaline phosphatase and serum bilirubin. All were studied before the start of treatment. A further patient who had classical anorexia nervosa was studied following weight regain

Controls The control subjects comprised 10 healthy young women aged 20–22 years. In all the observed/standard weight ratio fell between 90 and 118

Assessment of relative adiposity Height was measured to the nearest 0 25 in (0 6 cm) and weight to the nearest 0 25 lb (0 11 kg). The ratio of observed to standard weight was calculated according to the weight-for-height standards of KEMSLEY *et al.* [7]. This ratio was found to correlate closely with relative adiposity as estimated by body-density measurement. Blood was obtained from both patients and control subjects between 9 and 10 am after a light breakfast. All rested for a minimum of 20 min before venepuncture.

Results

The mean results for the control women and the individual results for the 7 patients with anorexia nervosa are presented in table I. The striking feature of the patients with anorexia nervosa is their high plasminogen activator activity, the mean level is significantly higher than that of the control women ($p<0.001$). A single patient who had regained weight after treatment was studied at the time her observed/standard weight ratio was 92 and found to have an activator level of 2.0 U, close to that of the mean of the control subjects. The mean levels of plasminogen and fibrin degradation products did not differ significantly from those of the normal women. However, the mean fibrinogen concentration of the anorexia nervosa patients was significantly below that of the control subjects ($p<0.005$). The levels of the major antiplasmins of human blood, α_1-antitrypsin and α_2-macroglobulin, did not differ significantly between the two groups.

Discussion

This study has demonstrated that patients with untreated anorexia nervosa have high plasminogen activator levels. The single patient studied after weight regain had an activator level in the range of the control subjects, suggesting that the changes in fibrinolytic activity in patients with anorexia nervosa are reversible and that they represent a response to chronic negative calorie balance similar to that found in healthy individuals subjected to short-term starvation. Notable is the lack of significant change in the levels of plasminogen or FDP in association with the high activator levels. The failure of plasminogen activation and the appearance of free plasmin activity within the circulation is characteristic of physiological increases in activator level [2].

The explanation for the lower fibrinogen concentration in the patients with anorexia nervosa is unknown. In the absence of a reduction in the plasminogen level or a rise in the FDP level, it is unlikely to be the result of intravascular proteolytic activity or coagulation. Fibrinogen production has been reported to be decreased by starvation when the diet is inadequate in proteins [3, 6]. However, evidence of protein deficiency in anorexia nervosa is minimal [15] and it is unlikely, therefore, that the lower fibrinogen levels are secondary to protein deficiency. It is possible that a relative expansion of the plasma volume, known to occur in anorexia nervosa [9], may contribute to the lowered fibrinogen concentration.

References

1 ALKJAERSIG, N, FLETCHER, A P, and SHERRY, S The mechanism of clot dissolution by plasmin J clin Invest 38 1086–1095 (1959)

2 BENNETT, N B; OGSTON, C M, and OGSTON, D The effect of prolonged exercise on the components of the blood fibrinolytic system J Physiol, Lond 198 479–485 (1968)

3 FOSTER, D P and WHIPPLE, G H · Blood fibrin studies II Normal fibrin values and the influence of diet Am J Physiol 58 379–392 (1922)

4 GRACE, C S · Fibrinolysis and obesity The effect of weight reduction Australas. Ann Med 18 32 35 (1969)

5 GRACE, C S and GOLDRICK, R B Fibrinolysis and body build. Interrelationships between blood fibrinolysis, body composition and parameters of lipid and carbohydrate metabolism. J Atheroscler Res 8 705–719 (1968)

6 HAM, T H and CURTIS, F C · Plasma fibrinogen response in man Influence of the nutritional state, induced hyperpyrexia, infectious disease and liver damage Medicine 17 413–445 (1938)

7 KEMSLEY, W F F, BILLEWICZ, W Z., and THOMSON, A. M A new weight for height standard based on anthropometric data Br J prev soc Med 16 189–195 (1962)

8 MANCINI G, CARBONARA, A O, and HEREMANS, J F · Immunochemical quantitation of antigens by single immunodiffusion. Int. J Immunochem. 2 235–254 (1965)

9 MANT, M F and FARAGHER, B S The haematology of anorexia nervosa Br J Haemat 23 737–749 (1972)

10 MERSKEY, C; KLEINER, G J, and JOHNSON, A J Quantitative estimation of split products of fibrinogen in human serum, relative to diagnosis and treatment Blood 28 1–18 (1966)

11 NILSSON, I M and OLOW, B Fibrinolysis induced by streptokinase in man Acta chir scand 123 247–266 (1962)

12 OGSTON, C M and OGSTON, D Plasma fibrinogen and plasminogen levels in health and in ischaemic heart disease J clin Path. 19 352–356 (1966)

13 OGSTON, D and MCANDREW, G M Fibrinolysis in obesity Lancet ii 1205 1207 (1964)

14 RATNOFF, O D and MENZIE, C A new method for the determination of fibrinogen in small samples of plasma J Lab clin Med. 37 316–320 (1951)

15 RUSSELL, G F M The nutritional disorder in anorexia nervosa J psychosom. Res. 11 41–49 (1967).

16 SHAW, D A and MACNAUGHTON D Relationship between blood fibrinolytic activity and body fatness. Lancet i 352–354 (1963).

17 SHERRY, S, LINDEMEYER, R I, FLETCHER, A P., and ALKJAERSIG, N Studies on enhanced fibrinolytic activity in man. J clin. Invest 38 810–822 (1959)

Dr D OGSTON, Department of Medicine, University of Aberdeen, Foresterhill, Aberdeen (Scotland)

Acta haemat 55 234–243 (1976)

Combined Congenital Deficiency of Factor V and Factor VIII

Report of a Further Case with Some Considerations on the Hereditary Transmission of this Disorder[1]

A. GIROLAMI, G. GASTALDI[2], G. PATRASSI and A. GALLETTI[2]

University of Padua Medical School, Institute of Semeiotica Medica, (Director Prof M. AUSTONI), Padua

Key Words Bleeding disorders · Combined congenital coagulation defects · Factor V deficiency · Factor VIII deficiency

Abstract A patient with combined factor V and factor VIII deficiency is presented. The bleeding manifestations were mild. The main laboratory feature was a prolonged partial thromboplastin time which was corrected by the addition of adsorbed normal plasma but not by the addition of normal serum, hemophilia A plasma or plasma of another patient with combined factor V and factor VIII deficiency. TGT was also clearly abnormal and was corrected by the addition of adsorbed normal plasma but not by the addition of normal serum. Prothrombin consumption was mildly defective. The prothrombin time was slightly prolonged. Factor VIII was 12% and factor V 55% of normal. Factor-VIII associated antigen was normal. The father and a sister of the propositus revealed mild factor V deficiency but normal factor VIII activity and antigen. The parents were not consanguineous. A tentative classification of combined deficiency of factors V and VIII in two groups is proposed. The hereditary transmission of the two types of deficiencies is discussed.

Combined deficiency of factors V and VIII is a rare coagulation disorder. Only 21 cases have been so far described in the literature [1, 10, 14–16, 18–21, 23–25].

The object of the present paper is to present another case with this peculiar defect. This is the 22nd case reported in the literature and the 4th in Italy. The other cases studied in Italy were reported a few years ago [10, 14]. Furthermore, this is the 3rd case for which a normal factor VIII antigen levels is recorded immunologically.

[1] This study was supported in part by a grant obtained from the CNR (grant 74.00189.04).

[2] Central Laboratory, Galliera Hospital, Genoa.

Case Report

The patient, a [redacted] year-old male, has been sent to us in February 1975 for an evaluation of his life long coagulation defect. He had been followed by one of us (G.G.) for the past 3 years. Family history was negative for bleeding disorders but a sister had undergone splenectomy for idiopathic thrombocytopenia. The parents were not consanguineous and came from different regions of Italy. The family pedigree is shown in figure 1. During infancy and childhood the patient presented no important bleeding manifestations. He had only easy bruising and occasional epistaxis.

At the age of 21, the patient started noticing occasional bleeding from the gums. At that time he was working in a printing company and appeared to be exposed to lead. A few months later, the bleeding was attributed to a mild thrombocytopenia even though slightly prolonged glass-clotting and recalcification times had been noted. A few months later, platelets were reported to be back to normal. At the age of 22, the patient presented excessive bleeding after two tooth extractions. On one occasion he had [redacted] be transfused with one unit of fresh blood.

At the age of 26, the patient presented a large hematoma of the left thigh after a minor trauma. He was admitted to a hospital in Genova where, because of leg varices, he was suspected [redacted] have a deep thrombophlebitis. However, a venographic study showed no deep vein thrombosis. The patient was treated with bed rest and the hematoma slowly subsided.

Altogether, bleeding manifestations have not been severe. During the last few years a tooth extraction, on two occasions, was followed only by moderate bleeding. At the time of study in 1975 there were no bleeding manifestations. All routine laboratory tests, including liver function studies, were within normal limits. Respiratory function studies revealed an obstructive pattern compatible with mild asthma.

Material and Methods

Material and methods have been dealt with in detail elsewhere [5-9]. Only new data will be given.

Anti factor VIII inhibitor activity was evaluated according to a modification of the method of BIGGS and BIDWELL [2]. A lyophilized factor VIII concentrate preparation (Kryobulin 250, Immuno, Wien) was reconstituted with 20 ml of distilled water according [redacted] to the manufacturer's instructions. 0.1 ml of this reconstituted factor VIII concentrate preparation was diluted 3-fold with normal saline. 0.1 ml of this preparation was then added [redacted] 0.9 ml of patient plasma or 'fake' plasma. The fake plasma was obtained by mixing 0.15 ml of normal plasma and 0.85 ml of hemophilia A plasma. The factor VIII level of the fake plasma was 15% of normal. After a 60-min incubation period at [redacted] °C, factor VIII was assayed in a one-stage system in both mixtures.

Factor VIII associated antigen was evaluated according to a previously reported modification of LAURELL's [11] method. Platelet adhesiveness was measured according [redacted] SALZMAN's [22] method.

Table I Coagulation study in the propositus

Test	Result	Normal values
Platelets/µl	170,000	150,000–350,000
Bleeding time, min	4	2–5
Clot retraction	complete after 10 h	complete after 12 h
Glass clotting time, min	10	5–9
Recalcification time, sec	260	100–180
Partial thromboplastin time, sec	69 2	35–45
Prothrombin consumption, %	70	>90
Thromboplastin generation test	30 sec in 8 min	19 sec in 6 or 8 min
Prothrombin time, sec	16	13–14
Stypven-cephalin clotting time, sec	14	10–12
PP test, sec	28 8	25–29
Factor II, %	100	85–115
Factor V, %	55	85–115
Factor VII, %	100	85–115
Factor VIII, %	12	60–160
Factor IX, %	100	85–115
Factor X, %	*110*	*85–115*
Factor XI, %	110	70–150
Factor XII, %	100	70–150
Fibrinogen, mg%	480	250–500
Fibrinolysis, h	15	10–30
Thromboelastogram. r, mm	26	10–20
K	12	6–12
ma	54	50–66
Thrombin time, sec	19	18–25
Platelet adhesiveness, %	32	20–50

Table II Partial thromboplastin time (PTT) correction studies

Mixture, equal parts	PTT of mixtures sec	PTT of reference sec
Propositus plasma	69 2	–
Propositus plasma + adsorbed normal plasma	37 5	–
Propositus plasma + normal serum	82	–
Propositus plasma + hemophilia A plasma	90	110
Propositus plasma + combined factor V and factor VIII deficiency plasma	76 6	72

Table III Lack of anti factor V and anti factor VIII activities in the patient's plasma on incubation at 37°C

	Incubation time min			
	0	30	60	120
Factor V level of a 1 2 mixture of patient's plasma and fresh normal plasma, %	105	96	86	80
Factor V level of a 1 2 mixture of aged normal plasma and fresh normal plasma, %	58	54	50	46
Kryoglobulin + propositus' plasma			74	
Kryoglobulin + fake plasma			70	

Table IV Factor V activity, factor VIII activity and factor VIII antigen in the family members of our propositus

Family of patient	Factor V %	Factor VIII %	Factor VIII antigen
Propositus	55	12	150
Mother	92	110	140
Father	60	100	120
Brother, II_1	110	105	120
Sister, II_3	55	110	160
Brother, II_3	105	110	115
Sister, II_4	100	120	90
Brother, II_5	96	100	110
Normal	85–120	60–160	60–160

Number after brothers and sisters of our propositus correspond to those on the genealogic tree on figure 1

Results

The results of the coagulation study are summarized in table I Routine tests revealed the presence of a first- and second-stage deficit Partial thromboplastin time (PTT) was clearly prolonged and was corrected by the addition of adsorbed normal plasma but not by the addition of normal serum, hemophilia A plasma or plasma of another patient with combined deficiency of factors V and VIII (table II)

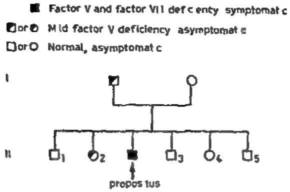


Fig 1 Family pedigree No paternal relative was available for study

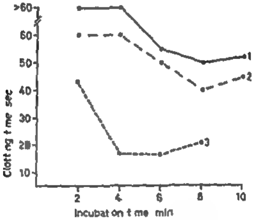


Fig 2 Thromboplastin generation test Curve 1 refers to the basal curve Curve 2 was obtained after the substitution of patient's serum with normal serum Curve 3 was obtained after the substitution of the patient's adsorbed plasma with adsorbed normal plasma The pattern of curve 3 is normal

The thromboelastogram showed a mild prolongation of r+K, whereas ma was within normal limits Prothrombin consumption was moderately defective The thromboplastin generation test was abnormal too and was corrected by the addition of adsorbed normal plasma (fig 2)

Factor V was 55% and factor VIII was 12% of normal Fibrinogen and factors II, VII, IX, X, XI and XII were all within normal limits Factor-VIII-associated antigen was normal No anti factor or anti-factor VIII inhibitors were present in the propositus plasma (table III) Platelet and vascular tests were within normal limits and there was no hyperfibrinolysis Platelet adhesiveness to glass was within normal limits too

Table V Tentative classification of combined deficiency of factors V and VIII

	Type I, pure association type	Type II, common gene abnormality	Comment
Frequency	rare (1)	common (1)	(1) on the basis of reported families
Consanguinity	*no*	*frequent*	
Factor V level	intermediate (2)	low (3)	(2) compatible with heterozygosis, clearly higher than factor VIII, (3) similar to factor VIII level
Factor VIII level	low	low	
Hereditary transmission	independent segregation of the two defects	autosomal, recessive	
Associated anomalies	no	frequent	

The father and one sister have mild factor V deficiency (table IV) Factor VIII was normal in all relatives studied both as activity and as antigen In particular, factor-VIII antigen versus factor-VIII activity was practically equal to 1 in all instances

Discussion

The clinical and laboratory features of this disorder appear to be well defined It is a first- and a second-stage defect characterised by a combined deficiency of factors V and VIII The only doubt remaining about the condition concerns its hereditary transmission

A few years ago, we have formulated the possibility that two types of combined deficiency of factors V and VIII may exist [19] The first group would consist of patients in whom a mild factor V defect compatible with heterozygosis is present together with hemophilia A In this case, a hemophiliac could have inherited one abnormal gene controlling factor V synthesis from either of the two parents No consanguinity is present between the parents The second type of defect would consist in an abnormality of one gene or a system of genes controlling a common step in the activation

of both factors V and VIII In this latter case, the condition appears to be transmitted as an autosomal recessive trait and consanguinity is frequently present between the parents One of the patients presented by GOBBI *et al* [14] is probably a typical example of the first type of defect The patients presented by SAITO *et al* [21], JONES *et al* [16], SELIGSOHN *et al* [24] and our previously reported case [10] belong to the second group The first group of patients could be termed type I or pure association type, whereas the second condition could be named type II The distinguishing features are summarized in table V

This tentative classification does not exclude the possibility that other variants of this disorder may exist For example, the family presented by SEIBERT *et al* [23] does not fit the above criteria In this case there was no consanguinity, the mother of the propositus had a factor V level compatible with heterozygosis whereas the factor V level of the propositus was definitely lower and similar to the factor VIII level

The present case, however, seems to confirm our hypothesis since the propositus appears to have a type I disorder Our patient is surely heterozygote for factor V deficiency since other family members have reduced factor V levels and there is no consanguinity between the parents In these cases, an independent segregation of the two defects may occur [14]

The crucial question of this type of combined factor V and VIII deficiency is whether the association is casual or, given a patient with heterozygous parahemophilia, a concomitant factor VIII defect is more likely to occur Parahemophilia is a rare coagulation disorder Only about 60 cases have been found so far in the literature Only 5 surely proven cases have been reported in Italy, together with approximately 20 heterozygotes [4, 7, 8, 16] Hemophilia A is surely a more frequent coagulation disorder About 3,000 hemophiliacs are suspected to be present in Italy Given a population of 55,000,000 that would indicate the presence of 1 hemophiliac in 20,000 The probabilities for a hemophilia A carrier to be also heterozygote for factor V deficiency are indeed very meager but may not be completely ruled out

The mother of our propositus does not seem to be a carrier This interpretation is supported by the factor VIII antigen versus factor VIII activity ratio and by the fact that three additional sons were perfectly normal Therefore, a mutation must be admitted, to explain the presence of a factor VIII defect in the propositus This behavior seems to be the rule in sporadic hemophilia

The existence of a normal factor VIII antigen level in these patients has been demonstrated in both instances in which it has been investigated [12, 26]. The demonstration of a normal antigen level in this third case too, seems to settle definitively the problem. Combined deficiency of factors V and VIII is associated with a normal factor VIII antigen and a low factor VIII activity. This is typical of a hemophilia like defect. It is interesting to note that in all cases studied, factor VIII antigen level was 100% or greater than 100% of normal. In this regard it may be worth noting that in combined deficiency of factors VII and VIII, a normal factor VIII antigen level was found too [3]. No information, on the contrary, is available for factor V. Is it a real deficiency or a structural defect whereby only activation is abnormal? The question can only be answered when satisfactory antifactor V antiserum is available.

The bleeding manifestations presented by our propositus have not been severe. Hemarthroses has never occurred in our patient but this is not unusual for mild hemophilia patients. In this regard, it may be noted that the factor V level may play an important role in the severity of bleeding. Our previous patient with this disorder had similar factor VIII levels but definitely lower factor V levels (18 versus 55%) than the present case and he had more important bleeding manifestations. The associated presence of leg varices in our propositus is probably only a casual association. The father of our propositus has leg varices too. However, the several associated anomalies such as hypogonadism, mental retardation, psychoneurotic or psychotic traits and enuresis which have been described in patients with this disorder [10, 15, 24]] should be remembered. These associated anomalies have been interpreted as an expression of consanguineous matings. Even in this regard, it may be noted that no associated anomaly has been described in the first group of patients (pure association disease).

References

1. Arspakis, G., Statiiakis, N., Papayannis, D., Thomopoulos, and Gorditos, C.: Familial study of a case with combined factor V and factor VIII deficiencies. Proc. 4th Congr. Int. Soc. Thromb. Haemost., p. 62 (1973).
2. Biggs, R. and Bidwell, E.: A method for the study of anti-haemophilic globulin inhibitors with reference to six cases. Br. J. Haemat. 5: 379 (1959).
3. De Vries, S. I.: Hemorragische diathese door tekort aan factor V (pro-accelerine). Ned. Tijdschr. Geneesk. 112: 741 (1968).
4. Girolami, A., Scarpa, R., Brunetti, A., and Lazzarin, M.: Congenital factor V deficiency in a 15 month-old child. Coagulation 2: 343 (1969).

of both factors V and VIII In this latter case, the condition appears to be transmitted as an autosomal recessive trait and consanguinity is frequently present between the parents One of the patients presented by GOBBI *et al* [14] is probably a typical example of the first type of defect The patients presented by SAITO *et al* [21], JONES *et al* [16], SELIGSOHN *et al* [24] and our previously reported case [10] belong to the second group The first group of patients could be termed type I or pure association type, whereas the second condition could be named type II The distinguishing features are summarized in table V

This tentative classification does not exclude the possibility that other variants of this disorder may exist For example, the family presented by SEIBERT *et al* [23] does not fit the above criteria In this case there was no consanguinity, the mother of the propositus had a factor V level compatible with heterozygosis whereas the factor V level of the propositus was definitely lower and similar to the factor VIII level

The present case, however, seems to confirm our hypothesis since the propositus appears to have a type I disorder Our patient is surely heterozygote for factor V deficiency since other family members have reduced factor V levels and there is no consanguinity between the parents In these cases, an independent segregation of the two defects may occur [14]

The crucial question of this type of combined factor V and VIII deficiency is whether the association is casual or, given a patient with heterozygous parahemophilia, a concomitant factor VIII defect is more likely to occur Parahemophilia is a rare coagulation disorder Only about 60 cases have been found so far in the literature Only 5 surely proven cases have been reported in Italy, together with approximately 20 heterozygotes [4, 7, 8, 16] Hemophilia A is surely a more frequent coagulation disorder About 3,000 hemophiliacs are suspected to be present in Italy Given a population of 55,000,000 that would indicate the presence of 1 hemophiliac in 20,000 The probabilities for a hemophilia A carrier to be also heterozygote for factor V deficiency are indeed very meager but may not be completely ruled out

The mother of our propositus does not seem to be a carrier This interpretation is supported by the factor VIII antigen versus factor VIII activity ratio and by the fact that three additional sons were perfectly normal Therefore, a mutation must be admitted, to explain the presence of a factor VIII defect in the propositus This behavior seems to be the rule in sporadic hemophilia

22 SALZMAN E. W. Measurement of platelet adhesiveness. J. clin. Lab. Med. 62 724 (1963)
23 SEIBERT R. H., MARGOLIUS A., and RATNOFF O. D. Observation on hemophilia, parahaemophilia and coexistent hemophilia and parahaemophilia. J. Lab. clin. Med. 52 449 (1958)
24 SELIGSOHN U. and RAMOT, B. Combined factor V and factor VIII deficiency. Report of four cases. Br. J. Haemat. 16 475 (1969)
25 SMIT SIBINGA C. T., GÖKEMEYER J. D. M., TEN KATE, L. P., and BOS VON ZWOL, F. Combined deficiency of factor V and factor VIII: report of a family and genetic analysis. Br. J. Haemat. 23 467 (1972)
26 ZIMMERMAN T. S., RATNOFF O. D., and POWELL, A. E. Immunologic differences of classic hemophilia (factor VIII deficiency) and von Willebrand's disease. J. clin. Invest. 50 244 (1971)

Prof. A. GIROLAMI, Istituto di Semeiotica Medica, Via Ospedale Civile 41, Padova (Italia)

Acta haemat 55 244–249 (1976)

Brucellosis in Two Thalassaemic Patients Infected by Blood Transfusions from the Same Donor

J Economidou, P. Kalafatas, T Vatopoulou, D Petropoulou and C Kattamis

Hellenic Red Cross Blood Research Laboratory, 2nd Propaedeutic Clinic of Athens University, Evangelismos Hospital, and Paediatric Clinic of Athens University, Children's Hospital, 'Agia Sophia', Athens

Key Words Brucellosis Thalassaemia Splenectomy Blood transfusion

Abstract Brucellosis was diagnosed in two polytransfused, thalassaemic patients who had received blood containing brucella agglutinins from an asymptomatic donor In one of the patients the onset of the disease simulated infectious mononucleosis Despite the fact that both cases were splenectomized, the course of illness was not life threatening However, a tendency for recurrence of the infection and persistence of high brucella agglutinin titres were noted

Brucellosis is a relatively common disease in many countries However, its transmission by blood transfusion must be extremely rare since very few cases have been reported in the literature Mena Brito [1] mentions 2 cases which were reported at the 2nd Mexican Brucellosis Congress in 1944 and reports 5 more instances of the disease transmitted from donors who had no indication of clinical brucellosis, but had brucella isolated from their blood afterwards In Mexico, Becerra-Garciá [2] added 3 cases from a personal communication by Castaneda The only case we could find in the British literature is that of Wood [3] who described the first case in Great Britain, diagnosis in both the donor and the recipient was based on a suggestive clinical picture and on a very high agglutinin titre, since the blood cultures were negative There are also few published details on the incubation time, symptoms and clinical course of brucellosis transmitted by blood transfusion

In this paper we report two cases of brucellosis occurring after transfusion of blood from the same donor who was found afterwards to have had brucella agglutinins in his serum at the time of blood donation. It is of interest that both patients were splenectomized cases of thalassaemia major. In one of the patients the onset of symptoms simulated an infectious mononucleosis-like syndrome. These patients attended the out-patient Thalassaemia Unit of the Hellenic Red Cross Blood Research Laboratory, for their regular transfusions and haematological follow-up.

Case Reports

Case 1. A 14-year-old girl suffering from homozygous β-thalassaemia had been subjected to splenectomy at the age of 10 for hypersplenism. She had been receiving regular transfusions since the age of 17 months and had had a total of 270 transfusions. On May 5, 1972 she experienced high fever for which she was admitted to hospital. Laboratory examination revealed a positive agglutination test for brucella at a titre of 1:2,500. On the basis of this finding, the diagnosis of brucellosis was made and a course of treatment with tetracycline and streptomycine was given. After an apyrexia of 40 days the patient, was readmitted to hospital for recurrence of symptoms. This time, blood cultures were positive for *Brucella melitensis* and the patient was given cotrimoxazol. After 6 and again after 10 months she experienced two more febrile attacks which were attributed to a relapse of brucellosis and responded to cotrimoxazol and cotrimoxazol plus streptomycine respectively. The patient was subsequently symptom free but died 1 year later from acute heart failure (table I).

This patient was living in Athens and had had no contacts with cattle, sheep or goats. The possibility of transmission of the disease by blood was considered and the sera of 4 donors who donated blood to her during a 4-month period before the onset of symptoms were examined afterwards for brucella agglutinins. One of the samples belonging to a donor who gave her blood on February 15, 1972 was found positive at a titre of 1:360 while the others were negative.

Case 2. A 17-year-old boy suffering also from homozygous β-thalassaemia had his spleen removed for hypersplenism at the age of 16. He was receiving regular transfusions since the age of 2 years and had had a total of 210 blood transfusions. On April 8, 1972 he complained of high fever of a week's duration. Physical examination showed hepatomegaly and enlarged cervical auxillary and inguinal lymph nodes of about 2 cm in diameter. His chest X-ray was normal. White blood cell count was 10,000/µl with metamyelocytes 1%, polymorphonuclears 16%, lymphocytes 40%, monocytes 19%, atypical basophilic lymphocytes 24%. The Paul Bunnell test was negative. The patient became apyretic after 1 week and the lymph node enlargement gradually subsided. On the basis of the haematological findings the clinical diagnosis of infectious mononucleosis was made. 2 months later he was admitted to hospital for high fever accompanied by chills, profuse sweating and prostration. The white blood cell count and differential count were

Table I Agglutination titres for brucellosis and clinical course of the donor and the affected patients

Case	Date	Agglutination titre for brucellosis	Clinical course
Donor	15 2 1972	1 320	healthy
	29 8 1972	1 160	healthy
Case 1	20 4 1972	1 2 560	*onset of fever*
	31 5 1972	1 320	relapse
	15 9 1972	1 640	symptom free
	6 12 1972	1 640	relapse
	7 3 1973	1 640	relapse
	28 9 1973	1 320	symptom free
Case 2	17 4 1972	<1 40	onset of fever
	29 6 1972	1 640	2nd febrile episode
	10 7 1972	1 1,280	on treatment
	29 11 1972	1 1,280	relapse
	17 5 1973	1 1 280	symptom free
	26 9 1973	1 320	symptom free

normal but a Wright agglutination test was positive to a progressively rising titre (table I) A blood culture was also positive for *Brucella melitensis* A serum sample dating from his first febrile episode was tested and found negative for brucella agglutinins The patient was treated with tetracycline streptomycine and cotrimoxazol for 3 weeks and was soon symptom free 4 months later he had a new febrile attack which was attributed to a relapse of brucellosis and was treated successfully with the same drugs He required no additional treatment and is well 2 years after his last febrile episode

The possibility of transmission of brucellosis to this patient by blood transfusion was again considered since no other source of infection could be implicated An investigation of the source of the transfused blood revealed that on March 23, 1972 the patient had received blood from the same donor with the positive brucella agglutination test as case 1

The donor A male farmer aged 26 who had contacts with cattle and goats gave blood for the first time in September 1971 to an unknown recipient On November 1971 he gave blood to a thalassaemic patient who experienced no illness subsequently Then followed two blood donations one on February 2 1972 and another on March 23, 1972 with recipients case 1 and 2 respectively His serum sample of February 2, 1972 was found to contain brucella agglutinins at a titre of 1 320 The donor was contacted again in August 1972 He was well with no abnormal physical signs and insisted that he had never been ill during the previous months or years He had also never been immunized against cholera His serum at that time had a brucella agglutination titre of 1 160

Discussion

Brucellosis is endemic in certain areas in Greece and the hazard of transmission of the disease by blood transfusion may be more common than suspected. According to a recent investigation [4] the frequency of positive serum tests in the general healthy population ranges in various areas from 1.7 to 12.8%. In the area of Larissa it was estimated that the frequency of brucellosis was 612 cases for 100 000 inhabitants per year. In the Hellenic Red Cross Thalassaemia Unit a total of 5 cases of brucellosis have been recorded during the past 5 years. All of these were thalassaemic polytransfused patients but unequivocal proof of transmission by blood was possible only for the two cases described in this paper. We consider that the high titre of brucella agglutinins in the serum of the donor at the time of blood donation to the two affected patients is highly indicative for active brucellosis. The lack of any clinical symptoms in the donor is not inconsistent with this concept since asymptomatic cases of brucellosis showing occasionally positive blood cultures have been reported [5]. Interesting in that respect are the cases of MENA BRITO [1] to whom brucellosis was transmitted by blood transfusion from asymptomatic donors.

At the University of Minnesota Hospital it was found that the blood of 27 (1.66%) of 1,627 apparently healthy donors whose blood was accepted for transfusion purposes had brucella agglutinins to a titre of 1:160 or higher [5], which is considered a significant diagnostic titre for active brucellosis [6]. It was further shown that representative Brucella organisms of the three species remained viable for as long as 6 months when the blood was refrigerated at 4 °C [5]. Yet, no instance of brucellosis transmitted by means of blood transfusion had ever been recognized at the University of Minnesota [6]. According to SPINK [6] except for a possible rare occasion it is doubtful that infection due to *Brucella* abortus can be transmitted by this route. It is however conceivable that infection might be initiated with the more invasive species of *Brucella melitensis*.

It is noteworthy that both our cases were splenectomized patients with thalassaemia major. It is well documented that thalassaemic patients after splenectomy are susceptible to severe and fatal infection from pneumococci, *Haemophilus influenzae* and *Escherichia coli* [7–9]. It appears that the clearance of certain microorganisms such as the pneumococci is defective in these patients [10]. Susceptibility to streptococcal infection has also been reported [11, 12]. It is therefore possible that splenectomized thalassaemic patients may have defective defence mechanisms against brucella trans-

mitted by the intravenous route It is remarkable that in both patients recurrence of the disease occurred despite adequate treatment and a high titre of brucella agglutinins was maintained for a long period after the control of active disease However, the infection was not life-treatening to either patient and responded promptly to antibiotics

Regarding the first pyrexial attack of case 2 which was initially attributed to infectious mononucleosis it is conceivable that this may represent the first febrile attack of brucellosis Lymph node enlargement and a blood picture with lymphocytoid cells similar to those found in infectious mononucleosis have been reported in brucellosis [6, 13–15] The absence of brucella agglutinins at this phase does not exclude this possibility because in brucellosis, several weeks or months may elapse before the appearance of agglutinins which in some rare instances may never develop [6, 16, 17] Because of this it may be difficult in some instances to differentiate brucellosis from infectious mononucleosis acquired by transfusion

Few relevant data exist in the literature concerning the incubation time of the brucella infection transmitted by blood transfusion The incubation time in case 1 was 50 days while in case 2 it could be either 8 days if the first pyrexial attack is to be considered as the onset of brucellosis or 60 days if the second one is considered as such

In this paper we would like to stress the importance of the hazard of brucella infection in polytransfused thalassaemic patients essentially in areas endemic for brucellosis The exclusion of subjects, known to have brucellosis from blood donation, as suggested by WHO [18] will not eliminate the hazard of transmission of the infection

References

1 Mena Brito, M A Brucellosis per transfusión sanguinea Primera Reunión Interamericana de la Brucelosis, p 707 (Ed del Hospital General, México 1948)

2 Becerra García, A Brucelosis y sífilis transmitidas por transfusión Gac méd Méx *101* 699 (1971)

3 Wood, E E Brucellosis as a hazard of blood transfusion Br med J *1* 88 (1955)

4 Papanajotou, P Symposium on endemic diseases, Larissa 1973 (in Greek), p 11

5 Spink, W W and Anderson, D Brucella studies on bank blood in a general hospital A Agglutinins, B Survival of brucella J Lab clin Med *35* 440 (1950)

6 Spink, W W The nature of brucellosis (University Minnesota Press, Minneapolis 1956)

7 Smith, C H, Erlandson, M R Stern, G and Hilgartner, M W Post splenectomy infection in Cooley s anemia Ann N Y Acad Sci *119* 748 (1964)

8 Eraklis A J, Kevy, S V, Diamond, L. K, and Gross, R E Hazard of overwhelming infection after splenectomy in childhood New Engl J Med *276* 1225 (1967)
9 Janeway, C. A Syndromes of diminished resistance to infection J Pediat *72* 885 (1968)
10 Schulkind M L., Ellis E F, and Smith R T Effect of antibody upon clearance of I[125] labeled pneumococci by the spleen and liver Pediat Res *1* 178 (1967)
11 Wasi P Streptococcal infection leading to cardiac and renal involvement in thalassaemia Lancet *i* 949 (1971)
12 Economidou J and Constantoulakis, M Streptococcal infection in thalassaemia Lancet *ii* 1160 (1971)
13 Kapsenberg J G Langenhuysen, M M A C, Nieweg H O, and Deiss, H Posttransfusion mononucleosis with heterophil antibodies Acta med scand *187* 79 (1970)
14 Wise H Acute brucellosis clinical bacteriologic and serologic studies of three patients Archs intern Med *72* 346 (1943)
15 Conway, H Lymphadenopathy in brucellosis Br med J *ii* 787 (1949)
16 Evans A C Chronic brucellosis J Am med Ass *103* 665 (1934)
17 Williams, E Brucellosis Br med J *i* 791 (1973)
18 Bowley, C C Goldsmith, K. L. G., and Maycock W D A Blood transfusion (W H O, Geneva 1971)

Dr J Economidou Blood Research Laboratory Hellenic Red Cross 4 Alkiviadou Street *Athens 179* (Greece)

Acta haemat. 55: 250-255 (1976)

Two Serum IgG-M Components of Different Light-Chain Types in a Case of Hodgkin's Disease

T. Chisesi, G. Capnist and T. Barbui

Divisione di Ematologia (Head: Prof. E. Dini), Ospedale Regionale, Vicenza

Key Words. Hodgkin's disease · Lymphomas · M components · Paraproteins

Abstract. The simultaneous occurrence of two serum and urine M components in a 75 year old woman affected by Hodgkin's disease is reported. The patient developed two IgG M components of different light chain types after an episode of pneumococcal pneumonia.

Monoclonal gammapathies associated with malignant lymphomas other than Waldenström's macroglobulinaemia or heavy-chain disease are infrequent. Moore *et al.* [7] studied the electrophoretic patterns of 333 patients with malignant lymphomas and found that 1.5% had monoclonal peaks. Comparing these with the incidence of similar monoclonal peaks in normal people, they concluded the occurrence of the IgG peak might be incidental and not related to the lymphoma, whereas the IgM monoclonal peaks occurred much too frequently to be an incidental finding.

In some cases of lymphoproliferative disorders, two M components have been reported [2–4]. Hobbs [4] and Seligmann and Basch [10] suggested that the appearance of the two M components reflected the presence of unstable, mutating cell populations. In Hodgkin's disease and nodular lymphomas, the incidence of monoclonal peaks has been found to be very low [5, 7]. The simultaneous occurrence of two serum and urine M components in a case of Hodgkin's disease is reported here.

Case Report

A 75 year old woman was admitted to our Division on February, 1972, for neck swelling noted 2 months before. We found enlarged lymph nodes only in the right

cervical region while thoracic roentgenograms and lymphography were negative. Lymph node biopsy was interpreted as Hodgkin's disease of nodular sclerosing type. Laboratory studies including bone marrow examination were normal. The Rye classification was used to determine the extent of the disease which was found to be stage I A. Between April 11 and 18, the patient received 4,500 mg Natulane i.v. and an appreciable improvement in cervical adenopathy was noted. She was seen again in June 1972 because of thoracic herpes zooster and was given 2.5 g γ-globulins i.v. per day as severe hypogammaglobulinaemia was detected.

During a bronchopneumonia occurring in the following month, the serum protein electrophoresis showed evident changes and in August a single M component was noted. In September and November, two distinct M components in the γ region were evident. The sternal bone marrow was normocellular: erythropoietic and granulocytopoietic lines were normally constituted. There was an adequate number of megacaryocytes. Plasma cells constituted about 5% of the elements and were not remarkable in morphology, lymphocytes were numerically and morphologically normal.

The terminal event was heart failure. She died in November 1972. Permission was not granted for autopsy.

Material and Methods

Serum protein concentrations were determined by the biuret method. Serum electrophoretic studies utilized cellulose acetate as supporting medium [1] and also included immunoelectrophoresis [9] with the use of antisera specific for IgG, IgA, IgM, κ- and λ-chains as well as rabbit antiserum against whole normal human serum. Immunoglobulins in serum were quantitated by the Mancini method, using Partigen from Behringwerke. Antisera were purchased from Behringwerke. Electrophoresis and immunoelectrophoresis were also performed on dialyzed urine. The urine was filtered and then dialyzed against polivinylpirrolidone for 2 days, using dialysis tubing with average pore size 24 Å.

Results

Serum protein electrophoresis and values of immunoglobulins are reported in figure 1. A rapid increase of IgG, without significant changes of IgA and IgM, was noted in a few months. Figure 2 shows the serum immunoelectrophoresis pattern, obtained with antiserum against whole normal serum and κ- and λ-chains. Figure 3 gives urine protein electrophoresis and figure 4 urine immunoelectrophoresis carried out with IgG antiserum. The urinary protein excretion was 300 mg/24 h. Bence-Jones protein was associated with global proteinuria.

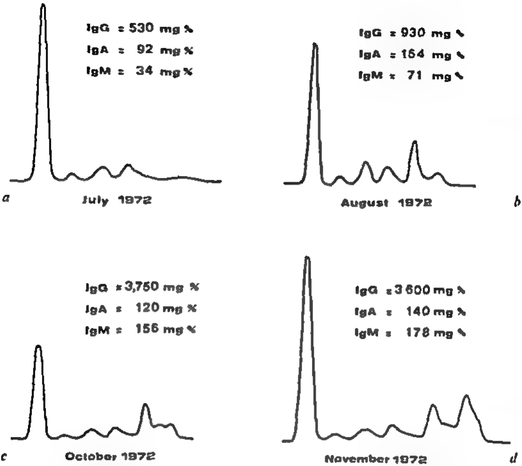


Fig 1 Serum protein electrophoresis carried out on different occasions *a* Hypogammaglobulinaemia *b, c* Single narrow based peaks *c* Two narrow based peaks

Discussion

This case showed the clinical picture of malignant lymphoma which is histologically classified as Hodgkin's disease of the nodular sclerosing type The protein abnormalities were observed during an episode of virus infection complicated with pneumonia and after intravenous administration of γ-globulins Cellulose acetate electrophoresis of serum examined in July showed hypogammaglobulinaemia whereas in August, during the acute phase of infection, a clear single serum M component was noted In the following months two serum M components gradually became evident and the IgG immunoglobulin values presented a rapid increase up to 3 750 mg% while IgA and IgM were within the normal range

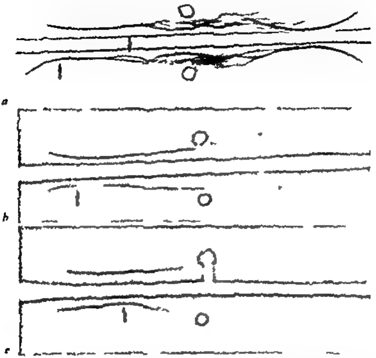


Fig. 2. Serum immunoelectrophoretic pattern. Normal serum above the well and patient's serum below the well. *a* Normal human antiserum. *b* κ *Antiserum.* *c* λ Antiserum. Arrows indicate the two IgG monoclonal components, the cathodic one of κ, the anodic one of λ type.

Serum immunoelectrophoresis performed with anti human whole serum in the sample obtained in November confirmed the two M components belonging to IgG immunoglobulins as shown with antiserum with IgG. Using anti κ and anti λ antisera, it was shown that the two M components were IgG κ and IgG λ. Urine immunoelectrophoresis revealed a light-chain excretion corresponding to the light chain of the serum IgG component.

There was nothing to support the diagnosis of myeloma which rarely has been reported to coexist with classic malignant lymphoreticular disease [6]. The bone marrow did not show myeloma cells nor Waldenström's disease tissue.

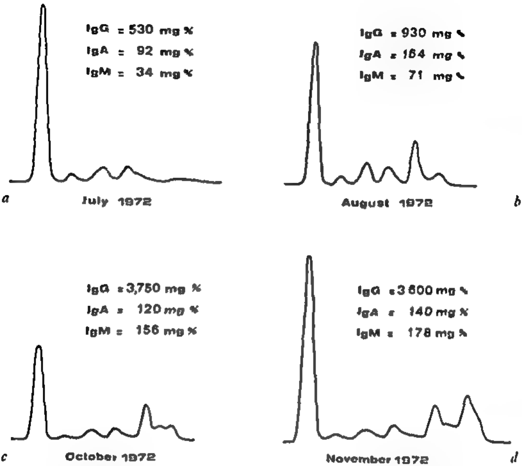


Fig 1 Serum protein electrophoresis carried out on different occasions a Hypogammaglobulinaemia b c Single narrow based peaks c Two narrow based peaks

Discussion

This case showed the clinical picture of malignant lymphoma which is histologically classified as Hodgkin's disease of the nodular sclerosing type The protein abnormalities were observed during an episode of virus infection complicated with pneumonia and after intravenous administration of γ-globulins Cellulose acetate electrophoresis of serum examined in July showed hypogammaglobulinaemia whereas in August, during the acute phase of infection, a clear single serum M component was noted In the following months two serum M components gradually became evident and the IgG immunoglobulin values presented a rapid increase up to 3,750 mg% while IgA and IgM were within the normal range

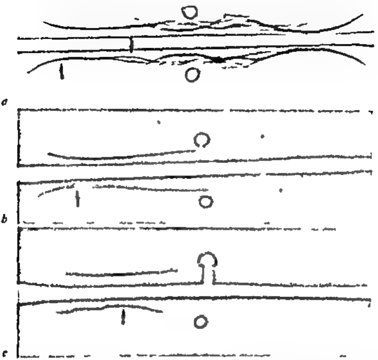
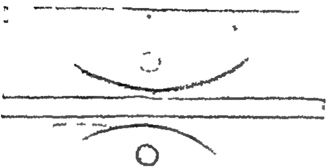


Fig 2 Serum immunoelectrophoretic pattern Normal serum above the well and patient's serum below the well *a* Normal human antiserum *b* $\varkappa$ Antiserum *c* λ Antiserum Arrows indicate the two IgG monoclonal components the cathodic one of $\varkappa$ the anodic one of λ type

Serum immunoelectrophoresis performed with anti human whole serum in the sample obtained in November confirmed the two M components belonging to IgG immunoglobulins as shown with antiserum with IgG Using anti $\varkappa$ and anti λ antisera it was shown that the two M components were IgG $\varkappa$ and IgG λ Urine immunoelectrophoresis revealed a light-chain excretion corresponding to the light chain of the serum IgG component

There was nothing to support the diagnosis of myeloma which rarely has been reported to coexist with classic malignant lymphoreticular disease [6] The bone marrow did not show myeloma cells nor Waldenstrom's disease tissue

3



4

Fig 3 Electrophoresis of urinary protein concentrate Arrows indicate two narrow bands

Fig 4 Immunoelectrophoresis on urinary protein concentrate, carried out with IgG antiserum Above normal, below patient s urine

Many investigators support the view that monoclonal protein originates in the neoplastic lymphoid cells [4–6] Others have noted either plasmocytosis or pyroninophilic lymphoid or reticulum cells, or both, in lymph nodes and bone marrow specimens from patients with lymphomas and have speculated that these, rather than the lymphoma, are the source of the abnormal globulin [11, 12] In addition, there are reports of patients with chronic lymphocytic leukemia who developed IgG-M components after an episode of pneumococcal pneumonia [3] or during a hypersensitivity reaction to drugs [8] These cases, as well as our case, presented a profound state of immunological deficiency and they could respond to immunological challenges with monoclonal or biclonal proliferation of immunoglobulin-producing cells

Acknowledgement The authors thank Dr F DAMMACCO for his helpful suggestions

References

1 Grunbaum B. W., Zec J., and Durrum E. L. Application of an improved micro-electrophoresis technique and immunoelectrophoresis of the serum proteins on cellulose acetate. Microchemistry *7* 41 (1963)

2 Hallen J. Discrete gammaglobulin (M-) components in serum: clinical study of 150 subjects without myelomatosis. Acta med. scand. *462* suppl., p. 1 (1966)

3 Hobbs J. R. Disturbances of the immunoglobulins. Sci. Basis Med. a. Rev *1966* 106–127

4 Hobbs, J. R. Paraproteins: benign or malignant? Brit. med. J. *ii* 699 (1967)

5 Krauss S. and Sokal J. E. Paraproteinemia in the lymphomas. Am. J. Med. *40* 400 (1966)

6 Maldonado J. E., Williams, R. C., Silverstein M. N., and Harrison E. G. Lymphosarcoma with Bence Jones Proteinuria (type *κ*) and IgG serum M-component (type *λ*): a bitypic gammapathy. Mayo clin. Proc. *45* 413 (1970)

7 Moore D. F., Migliore P. J., Shullenberger C. C., and Alexanian R. Monoclonal macroglobulinemia in malignant lymphoma. Ann. intern. Med. 72 43 (1970)

8 Osserman E. F. and Fahey J. L. Plasma cell dyscrasia: Current clinical and biochemical concepts. Am. J. Med. *44* 256 (1968)

9 Scheidegger J. J. Une micro-méthode de l'immunoélectrophorèse. Int. Archs Allergy appl. Immun. *7* 103 (1955)

10 Seligmann M. and Basch A. The clinical significance of pathological immunoglobulins. 12th Congr. Int. Soc. Haemat., New York 1968 pp. 21–31

11 Wood T. A. and Frenkel E. P. An unusual case of macroglobulinemia. Archs intern. Med. *119* 631 (1967)

12 Young J. K. and Schmidt F. C. H. Malignant lymphoma. A case with plasmocytosis and hyperglobulinemia. J. med. Soc. N. Y. *61* 479 (1964)

Dr. T. Chisesi, Divisione di Ematologia, Ospedale Regionale, Vicenza (Italia)

Deutsche Gesellschaft für Hämatologie

Die 19. Tagung der Deutschen Gesellschaft für Hämatologie findet in Freiburg/Breisgau vom 10. bis 13. Oktober 1976 statt. Kongress-Präsident: Prof. Dr. G. W. LÖHR, Freiburg.

Themen: Eisenstoffwechsel, Arzneimittelbedingte Anämien.

Auskunft: Priv.-Doz. Dr. H. ARNOLD, Medizinische Universitätsklinik, Hugstetterstrasse 55, *D-78 Freiburg/Breisgau* (BRD).

Pappenheim-Preis 1975

Anlässlich der gemeinsamen Tagung der Deutschen und Österreichischen Gesellschaft für Hämatologie in Bad Nauheim wurde der Pappenheim-Preis 1975 am 29. September 1975 Herrn Priv.-Doz. Dr. med. RUPERT ENGELHARDT (Medizinische Universitätsklinik Freiburg/Breisgau) für die Arbeit «Zum Aggregationsverhalten von Proteinen der Erythrozyten-Membran» verliehen.

Pappenheim-Preis 1976

Die Deutsche Gesellschaft für Hämatologie schreibt für 1976 erneut den Pappenheim-Preis aus. Der Preis, in diesem Jahre mit DM 3000.– dotiert, ist eine Auszeichnung für eine hervorragende deutschsprachige wissenschaftliche Arbeit, die sich mit klinischen, experimentellen oder theoretischen Fragen der Hämatologie befasst. Der oder die Autoren sollten nicht älter als 40 Jahre sein. Die Manuskripte können bis zum 31. 5. 1976 beim Sekretär der Gesellschaft eingereicht werden: Dr. med. K. P. HELLRIEGEL, Medizinische Universitätsklinik, Joseph-Stelzmann-Strasse 9, *D-5000 Köln 41*. Die Preisverleihung erfolgt während der Eröffnungssitzung der nächsten Tagung der Deutschen Gesellschaft für Hämatologie, die vom 10. bis 13. Oktober 1976 in Freiburg/Breisgau abgehalten wird.

Acta haemat 55 257-264 (1976)

Traitement des stades cliniques I et II de maladie de Hodgkin

Résultats obtenus chez 100 malades par l'association à la radiothérapie d'un ou deux cycles de chimiothérapie

C Lagarde, J Chauvergne, B Hœrni, J Touchard
M Durand, G Hœrni Simon et R Brunet

Fondation Bergonié Bordeaux

Key Words Combined chemotherapy radiotherapy of Hodgkin's disease Hodgkin's disease Lymphogranulomatosis

Abstract The results obtained by chemo+radiotherapy in 100 patients with Hodgkin's disease at stage I or II as defined by clinical and radiological staging are analysed All patients were treated by chemotherapy followed by radiotherapy, 74 received a complement chemotherapy after the radiotherapy Statistical analysis emphasizes the usefulness of associating two cycles of chemotherapy with the classical radiotherapy With this treatment which gives a constant remission rate of about 90% a long term chemotherapy is no longer necessary and the interest of exploratory laparotomy becomes negligible

La valeur d'une radiothérapie pour guérir les maladies de Hodgkin apparemment localisées a été bien établie il y a 10 ans lors du Symposium de Paris [9] et n'est pas discutée Nous l'avons appliquée régulièrement dès 1965 en obtenant les mêmes résultats que d'autres équipes Cependant, nous avons été conduits assez rapidement à reconnaître ses insuffisances, à analyser ses échecs concernant notamment les formes à déplétion lymphoïde [6] et dans ces conditions à lui ajouter un supplément de traitement sous forme d'une association chimiothérapique Dès 1967, nous avons soumis les malades présentant des éléments pronostiques péjoratifs (présence de signes généraux et en particulier, de fièvre [3] type histologique défavorable, tumeurs volumineuses notamment) à un ou deux cycles de chimiothérapie en plus de la radiothérapie classique Une analyse préliminaire (non publiée) ayant mis en évidence que les résultats ainsi obtenus étaient meilleurs que ceux enregistrés avec la radiothérapie isolée pour les malades à pronostic favorable et la tolé-

rance à l'ensemble de la séquence thérapeutique paraissant très acceptable, nous avons généralisé ce protocole de traitement pour tous les malades. C'est un bilan des résultats obtenus chez les 100 premiers malades ainsi traités que nous exposons ici.

Malades

De début 1967 à fin 1974, 100 malades pour lesquels le diagnostic de maladie de Hodgkin a pu être porté et vérifié sur un document histologique indiscutable et qui présentaient un stade I ou II (ganglionnaire d'un seul côté du diaphragme) après bilan clinique et radiographique ont reçu, en plus de la radiothérapie segmentaire, une chimiothérapie complémentaire. Le bilan préthérapeutique réalisé a été celui préconisé à l'issue du Symposium de RYE [7]. Il n'a été fait aucune laparotomie exploratrice.

Cette série comprend 66 hommes et 34 femmes dont l'âge varie entre 6 et 77 ans. Les principaux caractères des malades et de leur maladie sont indiqués par le tableau I.

Au début de la période considérée, seuls les malades présentant un ou plusieurs éléments de pronostic péjoratif ont été soumis à ce protocole. Puis, à partir de 1972, tous les malades présentant un stade I et II l'ont reçu sauf 3 : 2 pour des raisons non médicales et une femme pour laquelle le diagnostic a été porté au sixième mois de grossesse.

Tous ces malades ont été régulièrement suivis ; aucun n'a été perdu de vue. Leurs dossiers ont été exploités courant février 1975.

Traitement

Ces 100 malades ont tous reçu, d'une part, la radiothérapie classique et d'autre part, un cycle de chimiothérapie préalable. Cette chimiothérapie dont les résultats ont été présentés par ailleurs [1] associe vinblastine, procarbazine, cyclophosphamide et méthylprednisolone selon le schéma indiqué sur la fig. 1. Les doses unitaires sont fixes sauf chez les malades pesant moins de 40 kg où elles sont réduites de moitié et c'est la durée conditionnant la dose totale qui est modulée en fonction de la surface corporelle et de la leucocytose. La durée du cycle ne dépasse jamais 21 jours et le traitement est arrêté quand le nombre des leucocytes atteint 2 000/µl. Pour 3 malades, on a administré non pas un mais deux ou trois cycles de chimiothérapie, à un mois d'intervalle, avant l'irradiation en raison de l'existence d'une tumeur volumineuse qui a paru insuffisamment réduite après un cycle pour pouvoir être irradiée dans de bonnes conditions.

Chez tous les malades, la radiothérapie a été commencée immédiatement après la fin de la chimiothérapie. Pour ceux présentant une atteinte médiastinale ou sus claviculaire, la radiothérapie recouvrant les territoires sus diaphragmatique en mantelet et lombo aortique a été administrée en un seul temps chez 58 malades, en deux temps chez les 13 autres avec un intervalle libre d'un mois. Elle a distribué

Tableau I Principaux facteurs pronostiques

	Nombre de malades		Nombre de malades
Hommes	66	Nombre de territoires atteints	
Femmes	34	1	22
Age, ans		2	37
0-19	20	3	23
20-39	48	4	8
40-59	21	5	3
60-79	11	Médiastin	
Histologie		Normal	39
I, à prédominance lymphoïde	14	Envahi	61
II, à sclérose nodulaire	38	Extension juxta ganglionnaire	13
III, à cellularité mixte	31	Signes de compression	11
IV, à déplétion lymphoïde	14	Signes généraux	
Inclassable	3	Absents	[illegible]
Siège		Présents	32
Sus-diaphragmatique	89	Diamètre de la plus grande masse tumorale cm	
Sous-diaphragmatique	11	< 5	48
		5-10	32
		> 10	10

en 4 semaines une dose de 4 000 rad dans les territoires ganglionnaires macroscopiquement envahis [illegible] 3 500 rad dans les territoires adjacents une surimpression de 500 1 000 rad a été réalisée sur un reliquat tumoral chez 5 malades Le territoire lombo-aortique a été irradié chez 82 malades

Un mois après la fin de la radiothérapie, un nouveau bilan a été réalisé pour apprécier la qualité de la rémission En cas de rémission complète les malades ont reçu un nouveau cycle de chimiothérapie identique au précédent dans les limites de leur tolérance 26 malades [illegible] ont pas reçu cette chimiothérapie de consolidation, 21 avant que cette attitude ne soit adoptée et généralisée 3 en raison d une leucopénie persistante [illegible] 2 en raison de complication intercurrente

Enfin, après cette deuxième série de chimiothérapie tous les malades ont été laissés sans traitement Aucun n a reçu de chimiothérapie d'entretien

Chez 1 malade, qui était porteur d'une importante splénomégalie l'ablation d'une rate de 920 g a été réalisée avant la chimiothérapie initiale

Tolérance

Dans l'immédiat la chimiothérapie initiale n a pas empêché [illegible] réalisation de la radiothérapie telle qu elle était prévue Cette irradiation a souvent été commencée

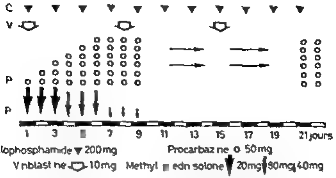


Fig 1 Schéma du protocole d association chimiothérapique

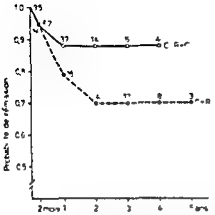
alors que le nombre des leucocytes était de l ordre de 2 000/µl et elle a été pour suivie dans la plupart des cas sans aggraver cette leucopenie Cependant chez 1 malade l irradiation a éte suspendue pendant 15 jours en raison d une leucopénie inferieure à 1 000/µl mais non compliquée avant d etre reprise et menée à terme Par ailleurs 1 malade de 76 ans est décédé dans les jours suivant l achèvement de la radiothérapie dans un tableau de septicémie à staphylocoques que nous avons rattachee au traitement chez les malades qui ont reçu une chimiothérapie de consolidation 4 aplasies medullaires ont été notées à son issue sévères mais reversibles apres une semaine d hospitalisation supplementaire Sauf pour le sujet décédé aucune de ces complications n a compromis l efficacite du traitement et tous ces malades sont actuellement en rémission persistante En fait loin d avoir compliqué la réalisation de l irradiation la chimiothérapie initiale paraît en avoir amélioré la tolerance grace à la réduction des dimensions des volumes irradiés [2]

En differe la responsabilité du traitement (irradiation et/ou association chimiothérapique) a été seulement suspectée pour une pneumonie fatale non spécifique verifiée à la necropsie Aucune autre complication n a éte notée Aucun deuxieme cancer n a eté observe A l exception de 2 tous les malades dont la maladie de Hodgkin a éte controlée ont repris leur activité professionnelle ou domestique antérieure

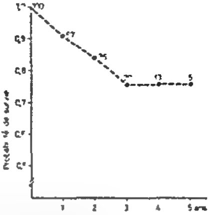
Résultats

Le but initial du traitement est de mettre le malade en *rémission complete* Ce resultat ne peut etre analyse pour 2 malades, l un decédé comme nous l avons vu à l issue du traitement, l autre 15 jours plus tard d hemorragies digestive intercurrentes Pour les 98 malades analysables la remission a ete complete dans 93 cas, incomplete dans 5 cas

Le *maintien de cette rémission complete* est indique par la figure 2 qui separe les malades ayant eu ou non une chimiothérapie de consolidation il n'y a plus de rechute au dela de 2 ans chez les malades n'ay-



2



3

Fig. 2. Courbes actuarielles des rémissions complètes obtenues après chimio+radiothérapie (C+R) et chimio+radio+chimiothérapie (C+R+C)
Fig. 3. Courbe actuarielle des survies non corrigées

ant reçu qu'un cycle de chimiothérapie et pour eux l'espérance de guérison se chiffre à 70%, et aucune rechute au-delà de 1 an chez ceux ayant reçu deux cycles de chimiothérapie et pour eux l'espérance de guérison atteint près de 90%.

Les 5 rémissions incomplètes à l'issue de la première séquence de chimiothérapie+radiothérapie ont été observées chez 4 hommes et 1 femme de 54, 50, 45, 39 et 20 ans, présentant une forme à prédominance lymphoïde pour l'un d'eux, à cellularité mixte et à sclérose nodulaire pour 2. Ce qui est surtout frappant c'est que 4 d'entre eux présentaient au départ des signes de compression médiastinale nette, pour le 5e, la rémission a été jugée incomplète à cause d'une vitesse de sédimentation anormalement élevée qui a été expliquée par une reprise évolutive hépatique ultérieure : ce malade est le seul pour lequel nous ayons pu regretter de ne pas avoir fait une laparotomie exploratrice.

Les 7 rechutes sont survenues chez 4 hommes et 3 femmes présentant une forme à déplétion lymphoïde (3), à sclérose nodulaire (2), à cellularité mixte (1) ou à prédominance lymphoïde (1). Tous, sauf 1, avaient une masse tumorale prédominante mesurant plus de 5 cm de diamètre.

Pour ces 12 échecs, la reprise ou la poursuite évolutive a été située dans les territoires irradiés pour 8 malades, en bordure de champ pour 2 et généralisée pour les 4 derniers.

La survie brute, portant sur les 100 malades, est indiquée par la figure 3. Les 15 décès enregistrés ont été liés à l'évolution hodgkinienne pour 9 d'entre eux, rattachés au traitement pour 1 et à des affections in-

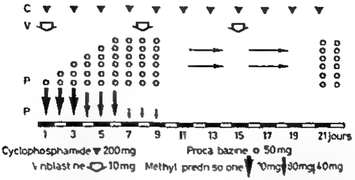


Fig 1 Schéma du protocole d association chimiothérapique

alors que le nombre des leucocytes était de l ordre de 2 000/µl elle a été pour suivie, dans la plupart des cas, sans aggraver cette leucopénie Cependant chez 1 malade, l irradiation a été suspendue pendant 15 jours en raison d une leucopénie inferieure à 1 000/µl mais non compliquee, avant d etre reprise et menée à terme Par ailleurs 1 malade de 76 ans est décédé dans les jours suivant l achevement de la radiothérapie dans un tableau de septicémie à staphylocoques que nous avons rattachée au traitement, chez les malades qui ont reçu une chimiothérapie de con solidation, 4 aplasies medullaires ont été notées à son issue sévères mais réversi bles après une semaine d hospitalisation supplémentaire Sauf pour le sujet décédé, aucune de ces complications n a compromis l efficacité du traitement et tous ces malades sont actuellement en rémission persistante En fait loin d avoir compliqué la réalisation de l irradiation la chimiothérapie initiale parait en avoir amélioré la tolerance grace à la réduction des dimensions des volumes irradiés [2]

En differé, la responsabilité du traitement (irradiation et/ou association chi miothérapique) a été seulement suspectée pour une pneumonie fatale non spéci fique, verifiée à la nécropsie Aucune autre complication n'a été notée Aucun deuxieme cancer n a été observé A l exception de 2, tous les malades dont la ma ladie de Hodgkin a été contrôlée, ont repris leur activité professionnelle ou domes tique antérieure

Résultats

Le but initial du traitement est de mettre le malade en *rémission complete* Ce resultat ne peut être analysé pour 2 malades, l'un décédé comme nous l'avons vu à l'issue du traitement, l'autre 15 jours plus tard d hemorragies digestive intercurrentes Pour les 98 malades analysables, la remission a ete complete dans 93 cas, incomplète dans 5 cas

Le *maintien de cette rémission complète* est indique par la figure 2 qui separe les malades ayant eu ou non une chimiothérapie de consoli- dation il n'y a plus de rechute au-delà de 2 ans chez les malades n'ay-

par rapport aux résultats obtenus avec la radiothérapie isolée [5]. Le bénéfice thérapeutique ainsi enregistré est acquis au prix d'une charge thérapeutique supplémentaire initiale mais aussi sans le traitement complémentaire de 2–3 ans par vinblastine souvent préconisé. Enfin, ces résultats sont obtenus sans avoir imposé aux malades de laparotomie exploratrice. Si cette intervention, qui permet de topographier avec plus de précision que le bilan radioclinique l'extension tumorale, est essentielle quand on réalise une radiothérapie exclusive, elle semble inutile en cas de chimiothérapie associée. Celle-ci permet d'atteindre les cellules néoplasiques où qu'elles soient et contribue ainsi, de façon décisive, à la guérison des malades.

Résumé

Depuis plusieurs années, nous traitons les malades atteints de maladie de Hodgkin aux stades cliniques I et II par une association radio-chimiothérapique; nous présentons ici les résultats obtenus chez les 100 premiers malades ainsi traités. Ils ont tous reçu d'abord un cycle de 2-3 semaines de polychimiothérapie d'induction immédiatement suivi de la radiothérapie. Trois quarts d'entre eux ont reçu ensuite un cycle de chimiothérapie de consolidation identique au premier. Aucun n'a reçu de traitement d'entretien. La qualité des résultats et leur stabilité (avec un plateau de rémission de l'ordre de 90% pour les malades ayant reçu deux cycles de chimiothérapie) indiquent que la laparotomie exploratrice comme la chimiothérapie d'entretien ne peuvent apporter, dans ces conditions, qu'un bénéfice supplémentaire minime; leur réalisation est donc inutile si l'on applique un tel protocole de traitement.

Bibliographie

1. Chauvergne, J., Hoerni, B., Hoerni-Simon, G., Durand, M. et Lagarde, C.: Chimiothérapie de la maladie de Hodgkin associant procarbazine, vinblastine, cyclophosphamide et méthyl-prednisolone. Analyse d'une série de 124 cures. Z. Krebsforsch. 80, 179–188 (1973).
2. Chauvergne, J., Touchard, J., Hoerni, B. et Durand, M.: Intérêt d'une chimiothérapie préalable à l'irradiation d'adénopathies médiastinales dans la maladie de Hodgkin (en préparation).
3. Hoerni, B., Chauvergne, J. et Parsi, M.: La fièvre de la maladie de Hodgkin. Hypothèse pathogénique. Presse méd. 78, 1317–1319 (1970).
4. Lagarde, C., Chauvergne, J., Durand, M., Hoerni, B., Hoerni-Simon, G., Le Treut, A., Meeus, C. et Touchard, J.: Survie des lymphomes malins. Analyse d'une série de 629 malades. Bordeaux méd. 8, 397–402 (1975).
5. Lagarde, C., Chauvergne, J., Durand, M., Hoerni, B., Hoerni-Simon, G. et Touchard, J.: Intérêt d'une chimiothérapie complémentaire de la radiothérapie dans les stades I et II de la maladie de Hodgkin. Bull. Cancer 62, 1–10 (1975).

6 LAGARDE C, TOUCHARD J, CHAUVERGNE J et HŒRNI B: Limites de la radiothérapie dans les formes réticulaires de maladie Hodgkin. J. Radiol. Electrol. *52*: 153–156 (1971).
7 ROSENBERG S A: Report of the committee on the staging of Hodgkin's disease. Cancer Res. *26*: 1310 (1966).
8 ROSENBERG S A and KAPLAN H S: The management of stages I, II and III Hodgkin's disease with combined radiotherapy and chemotherapy. Cancer Philad. *35*: 55–63 (1975).
9 Symposium: La radiothérapie de la maladie de Hodgkin. Nouv. Revue fr. Hémat. *6*: 1–176 (1966).

Prof. B. HŒRNI, Fondation Bergonié, 180, rue de Saint-Genès, *F–33076 Bordeaux Cédex* (France)

Acta haemat. 55: 265–271 (1976)

Pathogenetic Aspects of Anemia in Long-Term Hemodialyzed Patients

LUIGI BUSCARINI

Medical Department, General Hospital, Fiorenzuola d'Arda

Key Words. Anemia in hemodialysis · Hemodialysis · Uremia

Abstract. The anemia of dialyzed uremic patients is due to several factors: loss of blood and proteins, increased destruction of erythrocytes, and alteration of erythropoietin feedback. In an anemic dialyzed patient the number of reticulocytes is normal while a sudden worsening of the anemia is followed by severe reticulocytosis.

Several factors contribute to anemia in a patient undergoing hemodialysis. The loss of blood is considerable: from 1.57 to 4.62 liters/year according to HOCKEN and MARWAH [7], about 10 ml per dialytic session (taking into consideration only the blood residual in the dialyzer) according to BROZOVICH *et al.* [1], from 2 to 4.5 liters year according to LINDSAY and KENNEDY [16]. The survival of erythrocytes in a dialyzed patient is reduced; the phenomenon is, at least in part, connected with the traumatism caused by the pumps used for the blood flux [11, 13]. Concurring factors may be a deficiency of folate lost through the dialyzer [6] and a deficiency of iron due to both blood loss and impaired absorption [2, 5]. However, KOMINAMI *et al.* [11a] have observed normal serum iron values in a group of dialyzed patients. According to LAWSON *et al.* [14] a deficient erythrocyte incorporation of ^{59}Fe exists. Transferrin values are constantly reduced [10, 23]. Finally, the reduction or absence of erythropoietin production appears to be of great importance in the genesis of anemia [5].

In the present paper we refer some personal data which, together with data acquired from other publications, we think can contribute to a better clarification of the problem.

Material and Methods

In 11 uremic patients undergoing hemodialysis twice a week with Kiil s kidney for a total of 22 h (table I) we checked hemoglobin erythrocytes and reticulocytes monthly serum iron once every 2 months transferrin (radial immunodiffusion method) and osmotic erythrocyte resistance every year In add tion we searched for siderocytes burr cells [22] and erythrocyte antibodies (d rect and indirect Coombs test and tests on trypsinized red cells) every year We studied the hemoglobin p c ture at the beginning of dialysis and after 2 years (electrophoresis on cellulose ace tate) We tested the erythrocytes for glucose 6-phosphate dehydrogenase (G 6-PD) [12] and for pyruvate kinase (PK) [1] after about 30 months of dialysis Bone mar row aspiration was carried out after 24 months of dialysis and in case No 1 3 4 5 6 and 8 it was repeated at the 48th 60th 46th 39th 41st and 72nd month of di alysis respectively In addition to the morphological observation we also studied the maturation curve and the mitotic index of the erythroblasts We also did Perls reac tion for iron and PAS staining

We studied erythrocytic morphology with the scanning microscope in 5 patients at the third year of dialysis on samples drawn both at the beginning and at the end of a dialytic session

Results

Only one of the patients (case No 2 with a polycystic kidney) has an almost normal blood picture, in a second patient (case No 10 suffering from secondary amyloidosis) anemia is very mild, in all other patients the anemia is evident but of varying seriousness The reticulocyte values are practically normal (table I) The hemoglobin electrophoresis gave normal results except in one patient (No 5) who has a trait of thalassemia drepanocytosis The serum iron values are at low normal limits, the transferrin is constantly reduced (table I) The values of erythrocyte G 6 PD are normal or at the low normal limits, PK values are at higher limits or greatly increased (table I) Siderocyte research has always been negative The osmotic erythrocyte resistance is slightly reduced in every patient Burr cells are present in every patient they vary from 7 to 16% in sam ples which we examined immediately, after an hour s incubation period in a damp clamp chamber more than 40% of the erythrocytes present echin ocytic transformation (which on the contrary is not present in test sam ples from normal subjects)

During a first period erythrocyte antibody research was always nega tive in one case (No 7) 31 months after the beginning of dialysis hemolytic anemia due to cold anti I antibodies was present The considerable worsening of the anemia caused severe reticulocytosis The regression of

Table I

Case No	Age years	Sex	Months of dialysis	Diagnosis	Transfusions	Hb %	RBC × 10³ µl	Reticulocytes, ‰	Serum iron µg%	Transferrin mg%	mU/10⁹ erythrocytes G-6-PD	mU/10⁹ erythrocytes PK
1	59	f	31	ch n	2[1]	42	2,200	11–14	80	160	107	423
						45	2,400		91	160		
2	32	f	48	p k	–	74	4 000	7–10	[illegible]	190	129	267
						80	4,500		96	210		
3	43	m	60	ch. p	4[1]	55	2,900	8–13	80	170	152	283
						60	3,300		[illegible]	190		
4	60	m	46	ch n	4[1]	55	3 000	7–14	71	170	146	433
						60	3,300		84	190		
5	47	m	39	ch n	–	53	2,700	7–10	70	160	106	319
						60	3,200		91	170		
6	35	m	41	ch n	6[1]	40	2,200	13–16	76	150	120	390
						42	2,400		94	190		
7	49	m	41	ch. n	8	38	1,800	10–15	80	170	111	360
						42	2,300		98	185		
8	21	f	72	ch n	16	18	1,900	12–17	66	160	174	369
						45	2,300		79	190		
9	64	m	10	ch n	–	48	2,400	9–13	66	–	–	–
						50	2,900		88			
10	44	m	48	am k	–	66	3,900	7–14	90	155	130	280
						72	4 000		104	170		
11	44	m	51	ch n	18+6[1]	38	1,900	8–16	73	160	–	–
						42	2,200		93	180		
									normal values		120	60
											240	220

ch n = Chronic nephritis; p k = polycystic kidney; ch p = chronic pyelonephritis, am k = amyloid kidney

[1] Indicates the transfusions carried out to replace acute blood losses. With regard to case No 7 the data do not refer to the hyperhemolytic period. Hb, RBC, reticulocytes, serum iron, transferrin: extreme values.

a

b

Fig 1 Erythrocytes in the scanning microscope *a* Tendency to spherocytosis × 1,900 *b* Superficial roughness × 4 900

hyperhemolysis after treatment with azothioprine determined the return to the starting values of both red cells and reticulocytes

The study of the bone marrow showed a normal erythropoiesis without any morphological changes The leucoerythropoietic ratio was increased (equal to 4 2) in patient No 8 and normal in all other patients The maturation curve is normal in all patients except cases No 4 and 8 in whom the basophil phases were prevalent The erythroblastic mitotic index was

normal in cases No 5 and 8, and low in all other cases (between 5 and 8‰) PAS staining gave negative results, the sideroblasts were normal

With the scanning microscope the erythrocytes showed a tendency to spheric deformation, fragmented formes, and shapes with evident superficial roughness (fig. 1) These alterations were identical in test samples drawn both at the beginning and at the end of a dialytic session

Discussion

In the examined patients only case No 2 shows no signs of anemia In all other patients anemia is present though variable In the pathogenesis loss of blood is according to our data [unpublished], a little inferior to that referred in other publications (from 1,600 to 3,000 ml year), it is however, enough to cause a latent iron deficiency We must remember that loss of blood causes a simultaneous loss of proteins with abnormal demands on the protein synthesis [3, 17] The constant hypotransferrinemia in dialyzed patients is probably due to such factors as well as to an insufficient diet

The signs of increased fragility of the erythrocytes could be ascribed to certain uremic poisons which cannot be dialyzed or only with difficulty [4] However, an important factor is the microtraumatism caused by the extracorporeal circulation particularly by the thin strands of fibrin, which constantly form in the dialyzer We must point out that in our patients dialysis was performed with an external arteriovenous shunt without using mechanical pumps for blood flow Mechanical destruction could set off autoimmune phenomena

The red cells of uremic patients do not show any significant metabolic alterations on the contrary an increase of organic phosphates has been observed due to an activation of anaerobic glycolysis which is probably secondary to a rise in plasmatic phosphates [8 15] Within this ambit the increase of Pk values, frequently observed by us can be included

We could not see any megaloblasts or changes of iron utilization in the bone marrow (normal sideroblasts) The erythroblastic mitotic index is reduced in the majority of cases As usual the erythroblasts are PAS-negative However KERR and HEILER [7] revealed a high percentage of PAS positive erythroblasts in patients who had died of uremia or were in the terminal stage These contradictory reports may depend on the different seriousness of the disease

In our patients, even in the presence of severe anemia, the number of reticulocytes remained normal, however, in case No 7 further worsening of anemia was followed by acute reticulocytosis An analogous phenomenon was described by SHAW and SHOLES [25] following a severe hemorrhage The observation that the anemia in a dialyzed patient shows a normal reticulocyte count and that reticulocytosis can, however, follow a further critical fall of red cells induces us to suppose a varying change of the erythropoietin feedback mechanism In support is the fact that the kidney is not the only site of erythropoietin production [19, 21] while it certainly induces a normal erythropoietin feedback mechanism [18] On the other hand the increase of 2,3-DPG and ATP of the erythrocytes between the dialyses [15, 23] may shift the oxygen dissociation curve of the hemoglobin rightward, providing an increased amount of oxygen to the tissues so as to 'deceive' the structures that produce erythropoietin

References

1 BROZOVICH B CATTEL W R COTTRALL M F GWYTHER M M MCMILLAN J M MALPAS J S SALSBURY A and TROTT N G Iron metabolism in patients undergoing regular dialysis therapy Br med J *1* 695–698 (1971)

2 BUCHER T and PFLEIDERER G Pyruvate kinase from muscle in COLOWICK and KAPLAN Method in enzymology vol I p 323 (Academic Press New York 1955)

3 BUSCARINI L Rigenerazione delle sieroproteine e delle sieroglicoproteine in conigli sottoposti a salasso Archs Sci med *110* 275–290 (1960)

4 GIOVANNETTI S CIONI L BALESTRI P L and BIAGINI M Evidence that guanidines and some related compounds cause haemolysis in chronic uremia Clin Sci *34* 141–144 (1968)

5 *GRAL, T and SCHROTH P Transfusions transferrin saturation and erythropo et ic activity in long term hemodialysed uremic patients* Am J med Sci *260* 230–236 (1970)

6 *HAMPERS C L STREIFF R* NATHAN D G SNYDER D., and MERRILL, J P Megaloblastic hematopoiesis in uremic and in patients on long term hemodialysis New Engl J Med *276* 551–554 (1966)

7 HOCKEN D G and MARWAH P K Iatrogenic contribution to anemia of chronic renal failure Lancet *I* 164–165 (1971)

8 HURT G A and CHANUTIN A Organic phosphate compounds of erythrocytes from individuals with uremia J Lab clin Med *64* 675–684 (1964)

9 KLEIN H O and HELLER A PAS positive erythroblasts in kidney diseases Acta haemat *37* 225 239 (1967)

10 KLUTHE R BAUMANN G BISCHOFF G und QUIRIN H Serumtransferrin und Eiweissernahrung bei chronisch intermittierender Hämodialyse Med Ernahr *12* 73 75 (1971)

11 Kominami N Lowrie E G, Ianhez, L. E., Swaren A, Hampers, C. L. Merrill, J P., and Lange R D The effect of total nefrectomy on hematopoiesis in patients undergoing chronic hemodialysis J Lab clin. Med 78 524-532 (1971)
12 Kornberg A and Horecker B L. Glucose-6-phosphate dehydrogenase in Colowick and Kaplan Method in enzymology, vol 1 p 323 (Academic Press New York 1955)
13 Kurtides, E. S Rambach W A., and Del Greco F Effect of hemodialysis in anemia of uremia. J Lab clin Med 63 469-479 (1964)
14 Lawson D H, Boddy K, King P C Linton A L, and Will G Iron metabolism in patients with chronic renal failure on regular dialysis treatment. Clin Sci 41 345-351 (1971)
15 Lichtman M A and Miller, D R Erythrocyte glycolysis, 2,3-diphosphoglycerate and adenosine triphosphate concentration in uremic subjects. Relationship to extracellular phosphate concentration J Lab clin Med 76 267 279 (1970)
16 Lindsay R M and Kennedy A C Dialyser and blood loss in regular dialysis therapy Proc EDTA 9 437 454 (1972)
17 Marinone, G Esposito S Buscarini L. e Mazza, L. La donazione del sangue Problemi di biologia e di fisiopatologia Trasf Sangue 8 167 290 (1963)
18 Marinone, G e Buscarini L. Nuovi contributi al problema dei rapporti tra rene e fattore eritropoietico Haematologica 49 145 160 (1964)
19 Mirand E. A Murphy G P Steeves R A Weber H W., and Retief, F P Extrarenal production of erythropoietin in man Acta haemat 39 359-365 (1968)
20 Nalts J P and Wittek M Erythropoiesis in anephric man. Lancet I 941 943 (1968)
21 Nafts J P and Wittek M Presence of erythropoietin in the plasma of one anephric patient Blood 31 249 251 (1968)
22 Nathan D G Oski F A. Sidel, V W, Gardner F H, and Diamond L. K Studies of erythrocyte spicule formation in haemolytic anaemia Br J Haemat 12 385-395 (1966)
23 Quirin H Jutzler G A Traut R Baumann G Bischof G und Kluthe R Serumtransferrin bei chronisch hamodialysierten Patienten unter kontrollierter diatetischer Eiweisszulage Klin Wschr 51 247 249 (1973)
24 Raich, P C Rodriguez J M Desai J N., and Shahidi N T Effect of hemodialysis on erythrocyte 2,3-diphosphoglycerate in patients with uremia Am. J med Sci 265 147 151 (1973)
25 Shaw A B and Smoles, M C Reticulocytosis in renal failure Lancet I 799 802 (1967)

Dr L Buscarini, Reparto Medicina Ospedale Civile *Fiorenzuola d'Arda* (Italy)

Acta haemat. 55: 272–276 (1976)

Erythroblastic Islands and Extra-Medullary Erythropoiesis in Chronic Myeloid Leukaemia

U. Sjögren

Department of Internal Medicine, University Hospital, Lund

Key Words. Anaemia in leukaemia · Erythroblastic islands · Extramedullary erythropoiesis · Leukaemia

Abstract. Aspirates from bone marrow, spleen and liver were morphologically analysed in 15 untreated patients with chronic myeloid leukaemia. Megaloblastic changes of the erythroblasts were found to be more common in the spleen and liver than in the bone marrow. A significant increase of 'erythroblastic islands', i.e. erythroblasts in contact with reticulum cells, were recorded in the leukaemia patients compared to 15 healthy controls. It is suggested that the presence of such formations may indicate an increased death rate of defective erythroblasts followed by phagocytosis into reticuloendothelial cells.

The successive development of anaemia and hepatosplenomegaly are regular clinical features in chronic myeloid leukaemia (CML). It has previously been demonstrated that haemolysis [2], haemodilution and spleen sequestration of erythrocytes [3–5] may contribute to the anaemia. A reduced differentiation of basophilic erythroblasts towards more mature cells has also been proposed [9, 11]. Mitotic and morphologic studies of the spleen and liver erythroblasts have given evidence for a more defective function of the erythropoietic pool in the extramedullary sites than in the bone marrow [9, 12, 13].

Leukaemic involvement of the erythroblasts in erythroleukaemia and acute granulocytic leukaemia has been shown in many investigations with various techniques [for review see 10]. It has been suggested that the abundance of erythroblastic islands in acute myeloid leukaemias with a high frequency of megaloblastoid erythroblasts may be signs of ineffective erythropoiesis [10].

The present work was undertaken to investigate whether the morphol-

ogy of the erythropoietic tissue of the spleen and liver might indicate some abnormalities in the extramedullary production of red cells in CML. In addition to this a comparison between the intra- and extramedullary erythropoietic tissue was justified, since previous investigations have indicated that the extramedullary erythropoietic pool forms a considerable contribution of erythroid precursors [2, 8, 9, 12–14]. Special interest was devoted to the abundance of erythroblastic islands in the bone marrow, spleen and liver of the CML patients. It has previously been suggested that the presence of such formations may indicate phagocytosis of erythroblasts into reticulum cells [9, 10], thus being a sign of increased death rate of abnormal erythroblasts.

Material and Methods

Patients. 15 untreated patients with a recent diagnosis of CML were examined. There were 10 men and 5 women with a median age of 51 years. No patient was in blast crisis. The WBC counts were 76 000–311 000.

Controls. 15 apparently healthy persons, 7 men and 8 women, with normal haematological data and normal erythrocyte sedimentation rate served as controls. Their age was 19–82 years (median 51).

Morphologic investigations. Bone marrow was obtained through sternal puncture and material from the liver and spleen through fine-needle aspiration biopsy as described by Söderström [14]. Aspirates from the three organs were obtained on the same occasion and smears were stained with May-Grünwald-Giemsa. In each smear 1 000 erythroblasts were examined and morphologically classified according to Heilmeyer and Begemann [7] and Sjögren [9]. Proerythroblasts and basophilic erythroblasts were then pooled into one group and denominated basophilic erythroblasts. The proportion of erythroblasts with a megaloblastic morphology was determined. When erythroblasts were found in contact with or apparently lying within a reticulum cell the formation was classified as an erythroblastic island, and the number of such formations per 1 000 erythroblasts was registered. It proved impossible to carry out a blind analysis of the smears from the bone marrow, spleen and liver, since the splenic pulp cells and the liver parenchyma cells revealed the origin of the smears.

Statistics. The results are expressed as median values and ranges. The Wilcoxon matched pairs signed ranks test and the Mann-Whitney U test were used to assert significance of the results.

Results

The proportion of basophilic erythroblasts was on average 37.8% (24.5–53.8) in the bone marrow, 38.4% (21.8–53.4) in the spleen and 40.0% (23.0–61.6) in the liver. The differences are not significant.

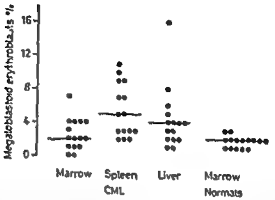


Fig 1 Percentage of erythroblasts with megaloblastic changes in the bone marrow, spleen and liver of 15 CML patients and in the bone marrow of 15 healthy controls Median values are indicated

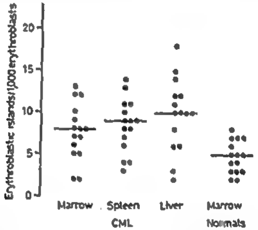


Fig 2 Frequency of erythroblastic islands in the bone marrow, spleen and liver of 15 CML patients and in the bone marrow of 15 healthy controls

The proportion of megaloblastoid erythroblasts was 2 1% in the bone marrow of the CML patients compared to 5 3% in the spleen The difference is significant ($p<0\ 02$) In the liver there were 3 8% megaloblasts This proportion is significantly higher than in the bone marrow ($p<0\ 05$) In the controls there were 1 7% megaloblasts in the bone marrow There is no significant difference between the bone marrow proportions of CML patients and the controls (fig 1)

The number of erythroblastic islands was 8/1,000 erythroblasts in the bone marrow, 9/1,000 in the spleen and 10/1,000 in the liver of the CML patients In the controls there were 5/1,000 erythroblasts in the bone

marrow. The difference between the CML patients and the controls is significant ($p<0.05$, fig. 2).

In the CML patients the number of erythroblastic islands was correlated to the percentage of basophilic erythroblasts within the erythropoietic pool, $r_s = 0.86$, $p<0.001$ in the bone marrow, $r_s = 0.82$, $p<0.01$ in the spleen, and $r_s = 0.94$, $p<0.001$ in the liver (the Spearman rank correlation coefficient).

Discussion

The present data indicate that the extramedullary erythropoiesis in CML obviously fails to compensate factors that promote the development of anaemia. Although previous investigations [8, 9, 12, 13] have demonstrated that there are higher proportions of erythroblasts in the extramedullary haemopoietic tissue than in the bone marrow, these erythroblasts have low mitotic indices indicating an impaired proliferative activity [9, 12, 13]. The composition of the erythropoietic pools of the intra- and extramedullary organs was probably the same with regard to erythroblasts at a different stage of maturation, since the proportions of basophilic erythroblasts were found to be the same in the bone marrow, spleen and liver. It has previously been proposed that the differentiation of early erythroblasts towards more mature erythroid cells becomes progressively blocked during the course of the disease, resulting in an accumulation of basophilic erythroblasts in the bone marrow of the CML patients [9, 11]. The present investigation gives evidence for a maturation arrest of the extramedullary erythroblasts as well as the erythroid cells of the bone marrow. However, megaloblastic changes, indicating a disturbed DNA synthesis, were more common in the extramedullary organs which is in agreement with previous observations [9, 12, 14]. Megaloblasts and disturbed DNA synthesis have also been reported in other morphological and biochemical investigations on CML [1, 6]. The mechanisms causing an impaired extramedullary production of erythroblasts are obscure. The discrepancies observed may be due to environmental differences between the bone marrow and the extramedullary organs.

Using various techniques some investigators have proposed that phagocytic reticuloendothelial cells may devour abnormal erythroblasts [for review see 9, 10]. The increased frequency of erythroblastic islands is probably the morphological sign of haemolysis. The abundance of erythroblastic islands in the extramedullary organs is in agreement with find-

ings of a latent haemolytic syndrome of the splenomegalic CML patients [2] The observations of an abnormally large porportion of basophilic erythroblasts and its correlation to the number of erythroblastic islands are compatible with an impaired differentiation towards more mature erythroid precursors An ineffective erythropoiesis with an increased death rate of erythroblasts within the haemopoietic tissue may, therefore, contribute to anaemia in CML as well as in the acute myeloid leukaemias

References

1 BART, J B Megaloblastosis in hematologic malignancy Sth med J, Nashville *66* 981–983 (1973)

2 BERLIN, R Red cell survival studies in normal and leukaemic subjects Acta med scand, suppl, p 252 (1951)

3 BLENDIS L M, RAMBOER, C, and WILLIAMS, R Studies on the haemodilution anaemia of splenomegaly Eur J clin Invest *1* 54–64 (1970)

4 BOWDLER, A J Blood volume studies in patients with splenomegaly Transfusion, Philad *10* 171–181 (1970)

5 CHRISTENSEN, B E Erythrocyte pooling and sequestration in enlarged spleens Estimations of splenic erythrocyte and plasma volume in splenomegalic patients Scand J Haematol *10* 106–119 (1973)

6 ERNST, P, BACCARANI, M, and KILLMANN, S A Biochemical abnormality of DNA synthesis of erythroblasts in acute and chronic myeloid leukemia Blood *41* 497–504 (1973)

7 HEILMEYER, L und BEGEMANN, H Atlas der klinischen Hämatologie und Cytologie (Springer, Berlin 1955)

8 MOESCHLIN, S Spleen puncture, pp 130–138 (Heinemann London 1951)

9 SJÖGREN, U Morphologic studies of haemopoietic tissue in myeloid leukaemias and megaloblastic anaemia, thesis Lund (1975)

10 SJÖGREN U Erythroblastic islands and ineffective erythropoiesis in acute myeloid leukaemia Acta haemat. *54* 11–17 (1975)

11 SJÖGREN, U and BRANDT, L Composition and mitotic activity of the erythropoietic part of the bone marrow in chronic myeloid leukaemia Scand J Haematol *12* 18–22 (1974)

12 SJÖGREN, U and BRANDT, L Differences in morphology and mitotic activity between intra- and extra medullary erythropoietic tissue in chronic myeloid leukaemia Scand J Haematol *13* 116–120 (1974)

13 SJÖGREN, U and BRANDT, L Different composition and mitotic activity of the haemopoietic tissue in bone marrow, spleen and liver in chronic myeloid leukaemia Acta haemat (in press)

14 SÖDERSTRÖM, N Fine needle aspiration biopsy Used as a direct adjunct in clinical diagnostic work (Grune & Stratton, New York 1966)

Dr ULF SJÖGREN, Department of Internal Medicine, University Hospital, *S-221 85 Lund* (Sweden)

Acta haemat. 55: 277–281 (1976)

Oxymetholone Effect on Acute Myeloblastic Leukemia Cells *in vitro*[1]

M. Ribas Mundo, J. G. San Miguel and C. Rozman

Escuela de Hematología 'Farreras Valentí', Hospital Clínico, Faculty of Medicine University of Barcelona, Barcelona

Key Words. Androgens · DNA synthesis · Leukemia · Oxymetholone

Abstract. The possibility that oxymetholone might induce or enhance leukemia after androgen therapy for aplastic anemia prompted us to study the direct action of oxymetholone on the DNA synthesis of AML cells *in vitro*. The peripheral blasts of 10 patients, 8 with AML and 2 with CML in blast crisis have been studied. The DNA synthesis of the leukemic cells with and without oxymetholone was measured by the ^{3}H methyl thymidine incorporation determined by liquid scintillation. The results have shown a wide variation of DNA synthesis from patient to patient with a range from 2 000 to 40 000 cpm but no significant difference between test and control cultures. We may conclude that oxymetholone does not increase directly the proliferation capacity of the peripheral AML cells cultured *in vitro*.

The paper by Delamore and Geary [5], reporting four patients who developed acute myeloblastic leukemia after treatment of aplastic anemia with oxymetholone, prompted us to study the direct action of oxymetholone on the DNA synthesis of acute myeloblastic leukemia (AML) cells *in vitro*.

Materials and Methods

Ten patients, eight with AML and two with CML in blastic crisis have been studied. The diagnosis of AML was based on the clinical findings and the cytological abnormalities observed in the peripheral blood smear and the bone marrow. All eight cases of AML were confirmed by cytochemical reactions. There was no case of promyelocytic or monoblastic leukemia, although patient 6 could be cytologically

[1] Read partially at the Second Meeting of the European and African Division of the International Society of Haematology, Prague 1973.

but not cytochemically considered as a myelomonocytic leukemia. None of the eight patients with AML had been previously treated, thus they all were in their first per ceptive phase of the disease

The two cases of CML in blastic crisis had both previously received Busulfan and their diagnosis had been established 2 and 4½ years before their first signs of blastic crisis In all cases, peripheral blast cells were higher than 80% except for the two cases of blastic crisis that were 58 and 35%

5 ml of venous blood were withdrawn into a sterile tube containing 0.5 mg he parin Cells were let to sediment at 37 °C for about half an hour, and the leukocyte layer was withdrawn Cultures were prepared with a final peripheral blast concen tration of 10^6/ml Culture medium 199 TC Difco was supplemented with 20% AB serum

Aqueous suspension of oxymetholone (provided by Syntex) at a final concentra tion of 3×10^{-6} M was added to the test cultures Test and control cultures were as sayed by addition of ^{3}H methyl thymidine 1 µCi/ml (spec act 1.9 Ci/mM) at the be ginning of the 4 hour cultures All cultures were done in duplicates DNA synthesis was measured according to DUPONT *et al* [6] as cpm/10^6 cells in a Beckman LS 250 liquid scintillation system

Results

The results have shown a wide variation of DNA synthesis from patient to patient with a range from 2,000 to nearly 40,000 cpm (table I) There is a tendency to lower values when the peripheral blast count is higher, but this is not true in all cases The lowest value was found in the cytologically considered acute myelomonocytic leukemia (case 6), but there was also a low value in cases 4 and 8 where we would expect a much higher thymidine incorporation The two patients with CML and blast crisis showed also divergent results with a very low incorporation in case 10 in spite of a reasonable number of blast cells and a very aggressive disease

We could not find any correlation between cpm and response to treatment Cases 1, 2, and 7 showed complete remission, case 4 had a partial remission and cases 3, 5, 6 and 8 showed no response to therapy Thus, cases with high and low thymidine incorporation showed no response, but the cases which responded had a tendency to higher values Both cases with CML and blastic crisis died without response

Our study shows no statistically significant differences between control and test cultures with oxymetholone In some cases, we even noted a slight decrease of thymidine incorporation Therefore, we may conclude that oxymetholone does not increase directly the proliferation capacity of the peripheral AML cells cultured *in vitro*

Table I Results of ^{3}H thymidine incorporation by peripheral blast cells cultured *in vitro* with and without oxymetholone

Case	Sex	Age years	Type	Peripheral blast count	Control cpm/10^4 cells	Oxymetholone cpm/10^4 cells
1	M	22	AML	86 000	20,368 19 866	19 957 17 603
2	M	[illegible]	AML	33 000	27,831 29,254	28,335 26 941
3	F	46	AML	142,000	3,310 3 913	2,858 3 709
4	M	65	AML	18 000	12,576 10 895	13 467 12,381
5	M	38	AML	21,500	39,232 36,779	37 628 38 922
6	M	59	AML	42,000	2,059 2,372	1 935 2,151
7	F	34	AML	97 000	16 744 17 657	16,582 16,938
8	M	37	AML	26,500	7,241 7 833	7,952 6,505
9	F	43	CML BC	29 000	22,079 24,286	23 670 21 432
10	F	61	CML BC	38 500	5 657 4 118	4,350 5,221

Discussion

We are aware of the limited values of these results as we have been only working with peripheral blast cells. It might be possible that the bone marrow blast cells would respond to oxymetholone, but there are some technical difficulties for standardizing the number and type of cells from bone marrow cultured *in vitro*. In our cases, the morphology of the peripheral blast cells was very similar to that of the blast cells in the bone marrow. If there is any leukemic cell sensitive to oxymetholone this would possibly be a leukemic precursor whose activation should be very difficult to detect by short term cultures. Other methods as colony forming unit cells either *in vivo* or *in vitro* might be used.

SHAHIDI [16] has recently summarized the mechanisms of action of androgens on the erythropoietic cells. The indirect action of androgens on erythropoiesis has been clearly established by many studies [1, 2, 9, 12], all showing consistently an increased level of endogenous erythropoietin. This indirect action should not by itself produce any effect on leukemic cells. On the other hand, several investigators have recently suggested a *direct* action of androgens on erythropoietic stem cells [8, 14]. Certain 5β-H-steroids appreciably stimulate porphyrin and hemoglobin synthesis [11, 13]. This effect is presumably achieved by stimulating δ-aminolevulinic synthetase, the rate-limiting enzyme for heme synthesis. Evidently, this type of direct action could not affect leukemic cells.

Some reports suggest that androgen metabolites trigger the colony-forming unit cells into the cell cycle [4, 15]. Thus, by triggering the pluripotential stem cells from G_0 phase or prolonged G_1 phase into a G_1 interval responsive to erythropoietin, a greater number of erythropoietin-sensitive cells would be available to the increased level of erythropoietin. This could be the only way by which androgen metabolites might affect directly acute leukemia cells or their precursor cells, and, at least in our study, we have not been able to demonstrate this direct effect on the peripheral acute leukemia blast cells.

Recently, the development of hepatocellular 'hepatoma' or 'hepatocarcinoma' in patients with aplastic anemia treated with androgens has been reported [3, 10]. Although there is no clear evidence of a definitive malignant transformation of normal hepatocytes by androgens, the clinical observation points to another possible deleterious effect of long-term androgen therapy. On the contrary, a recent report [7] suggests that androgen might trigger cells into cycles that respond to differentiating factors (erythropoietin), thus keeping the patient in a controlled preleukemia stage.

Acknowledgments. This investigation was supported in part by a research grant to M. R. M. *from the Juan March's Foundation.* We wish to thank Miss F. PUENTE for her technical assistance.

References

1 ALEXANIAN, R.: Erythropoietin and erythropoiesis in anemic man following androgens. Blood *33:* 564 (1969).
2 ALEXANIAN, R.; NADELL, J., and ALFREY, C.: Oxymetholone treatment for the anemia of bone marrow failure. Blood *40:* 353 (1972).
3 BERNSTEIN, M. S.; HUNTER, R. L., and YACHNIN, S.: Hepatoma and peliosis hepa

titis developing in a patient with Fanconi's anemia. New Engl. J. Med. 284 1135 (1971)
4 Byron, J. W. Effect of steroids on the cycling of haemopoietic stem cells. Nature Lond. 228 1204 (1970)
5 Delamore, I. W. and Geary, C. G. Aplastic anaemia, acute myeloblastic leukaemia and oxymetholone. Br. med. J. II 743 (1971)
6 Dupont, B., Nielsen, L. S., and Svejgaard, A. Relative importance of four and LA loci in determining mixed lymphocyte reaction. Lancet II 1336 (1971)
7 Gorshein, D., Besa, E. C., and Gardner, F. H. Acute myelomonocytic leukemia after cessation of androgen therapy. Ann. intern. Med. 79 749 (1973)
8 Granick, S. and Kappas, A. Steroid control of porphyrin and heme biosynthesis: a new biological function of steroid hormone metabolites. Proc. natn. Acad. Sci. USA 57 1463 (1967)
9 Gurney, C. W. and Fried, W. Further studies on the erythropoietic effect of androgens. J. Lab. clin. Med. 65 775 (1965)
10 Johnson, F. L., Lerner, K. G., Siegel, M., Feagler, J. R., Majerus, P. W., Hartmann, J. R., and Thomas, E. D. Association of androgenic anabolic steroid therapy with development of hepatocellular carcinoma. Lancet ii 1273 (1972)
11 Levere, R. D., Kappas, A., and Granick, S. Stimulation of hemoglobin synthesis in chick blastoderms by certain 5β androstane and 5β pregnane steroids. Proc. natn. Acad. Sci. USA 58 985 (1967)
12 Naets, J. P. and Wittek, M. Mechanism of action of androgens on erythropoiesis. Am. J. Physiol. 210 315 (1966)
13 Necheles, T. F. and Rai, U. S. Studies on the control of hemoglobin synthesis: the *in vitro* stimulating effect of a 5β H steroid metabolite on heme formation in human bone marrow cells. Blood 34 380 (1969)
14 Reisner, F. H., jr. Tissue culture of bone marrow. II. Effect of steroid hormones on hematopoiesis *in vitro*. Blood 27 460 (1966)
15 Reissmann, K. R. and Ito, K. Selective inhibition of erythroid cell differentiation in the mouse by low doses of actinomycin D. Ann. N.Y. Acad. Sci. 149 193 (1968)
16 Shahidi, N. T. Androgens and erythropoiesis. New Engl. J. Med. 289 72 (1973)

Prof. M. Rozman Mundo, Facultad de Medicina, C. Casanova 143, *Barcelona 11* (Spain)

Acta haemat 55 282–288 (1976)

Glycopeptides of Erythrocyte Membranes in Some Hematological Disorders

C. L. BALDUINI, M E TIRA, E ASCARI and C. BALDUINI

Institute of Clinical Medicine A Ferrata Faculty of Medicine and Surgery, and Institute of Biological Chemistry, Faculty of Sciences, University of Pavia Pavia

Key Words Dyserythropoietic anemia Erythrocyte membrane Glycopeptides PNH Polycythemia vera Sialic acid

Abstract Red cell membrane glycopeptides of subjects suffering from different hematological disorders (PNH hemolytic anemias, dyserythropoietic anemias and polycythemia vera) have been characterized In most cases except in polycythemia vera a decrease of sialic acid and galactosamine was detected The role of these alterations is discussed with regard to the decrease of membrane glycopeptides during physiological aging of the red cell

Many authors have investigated the essential role of glycoproteins located at the external surface of the erythrocyte membrane [7, 10, 17–19] Sialic acid, a component of glycoproteins, was studied in particular and removal of this component from the membrane modifies some properties of the erythrocyte, like agglutination by viruses [11], antigenic characteristics [14], and life span [5, 6]

In our institute it has recently been shown that during the aging of erythrocytes in circulation, sialic acid and galactosamine are removed from membrane glycoproteins [1] On the basis of this evidence it was decided to investigate if the modifications which appear during physiological aging might also be present in some hematological disorders characterized by shortened red cell survival Erythrocyte membranes of patients suffering from paroxysmal nocturnal hemoglobinuria (PNH) were also studied considering that normal erythrocytes after neuraminidase treatment are similar to PNH cells and in particular become susceptible to the hemolytic action of serum complement [20] A case of polycythemia vera was

also investigated on the basis of the hypothesis that in this disease a higher degree of young erythrocytes is present in the circulation [2].

Materials and Methods

We studied glycopeptides extracted from erythrocyte membranes in 2 patients affected by congenital dyserythropoietic anemia type II (HEMPAS, cases 1, 2) and in 4 patients with sideroblastic anemia (cases 3–6). The red cell survival time was shortened in all these patients. Two cases of PNH were also studied (cases 7, 8). These patients had no severe hemolytic episode in the last 2 weeks. We also investigated erythrocyte membranes in 1 patient affected by polycythemia vera (case 9) and in two brothers, 43 and 41 years old, suffering from muscular dystrophy and severe hemolytic anemia from birth (cases 10, 11). The clinical features were similar in both cases: apart from pallor and mild splenomegaly no physical abnormalities were present. Laboratory investigations showed: (a) considerable normochromic normocytic anemia with a slightly elevated reticulocyte count; (b) no intrinsic or extrinsic red cell abnormalities could be demonstrated; (c) in particular, the enzymes of glycolysis, hexosemonophosphate shunt and other clinically significant enzymes were within the normal values; (d) no pathological hemoglobins were detectable, and (e) warm and cold antibodies were not present. The red cell survival was slightly shortened. All patients had no therapy or blood transfusion in the last month. The reticulocyte count was normal in cases 3, 4 and 9 and increased in the other patients (14% in case 1, 20% in 2, 11% in 5, 12% in 6, 18% in 7, 12% in 8, 9% in 10 and 10% in 11).

Preparation of red cell ghosts. A slight modification of the method of Dodge *et al.* [4] was used. 40–50 ml of blood were collected from the veins of patients using 3.8% sodium citrate as anticoagulant. Plasma and buffy coat (white cells) were removed after centrifugation at 4 °C at 1,000 *g* for 15 min and erythrocytes were washed three times with 10 vol of isoosmotic (310 mosm) sodium phosphate buffer pH 7.4. Packed erythrocytes were then suspended in 4 vol of 20 mosm sodium phosphate buffer, pH 7.4 and maintained at 4 °C for 12 h. Ghosts were then sedimented by centrifugation at 20,000 *g* at 4 °C for 40 min and washed with 20 mosm sodium phosphate buffer, pH 7.4, until the supernatant was colorless, to remove as much hemoglobin as possible. A partial lipid extraction was carried out by suspending the sediment in 10 vol of ethanol-diethyl ether (3:1, v/v) kept stirring for 5 h at 4 °C [12]. The treatment was repeated twice and the ghosts were then washed with 99% (v/v) ethanol and then ether.

Extraction of glycopeptides from red cell ghosts. Glycopeptides were extracted from membranes by papain digestion (1 U of papain/mg of dry ghosts) in 0.1 M sodium acetate buffer, pH 5.5, containing 5 mM EDTA and 5 mM cysteine at 65 °C for 45 h [13]. After heat inactivation of the enzyme and removal of the insoluble particles, the solution was dialyzed against bidistilled water and glycopeptides were precipitated in the presence of 0.6 M sodium acetate and 0.5 M acetic acid at 4 °C by the addition of 3 vol of 99% (v/v) ethanol [1]. No sialic acid was detectable in the supernatant.

Table I Sialic acid and hexosamine content in glycopeptides extracted from erythrocyte ghosts of 6 patients affected by dyserthropoietic anemias

	Normal values		Dyserythropoietic anemias					
	young ghosts	old ghosts	HEMPAS		acquired			
			1	2	3	4	5	6
Sialic acid	13.7±1.2	9.9±2.1	traces	3.0	7.5	4.1	8.3	13.7
Galactosamine	5.1±0.1	4.4±0.1	traces	2.0	2.5	2.4	ND	7.6
Glucosamine	13.2±1.9	14.0±2.4	13.2	10.9	11.5	21.7	ND	15.2

Normal values are means ± SD of 4–6 experiments carried out on pools of 2–3 donors each [1]. Values are expressed as g/100 g dry weight.

Analytical methods. Hexosamines were determined with the Hitachi Perkin Elmer Liquid Chromatograph [9]. Sialic acid was measured by the method of SVENNERHOLM [15], hexoses by the method of TREVELYAN and HARRISON [16], fucose by the method of DISCHE and SHETTLES [3] and total protein by the method of LOWRY *et al.* [8].

Results

Dyserythropoietic anemia. Table I shows a significant decrease of sialic acid in three cases of acquired dyserythropoietic anemia, galactosamine concentration is also lower than in normal ghosts in cases 3 and 4, while glucosamine content shows considerable variations. In a fourth patient (No. 6) with acquired dyserythropoietic anemia the levels of sialic acid, glucosamine and galactosamine were among the normal values. The determination of fucose, hexose and protein was carried out only on glycopeptides of case 3, owing to the poor blood availability of the three other patients, and did not show any variation from normality. Protein and sugar composition was also normal in glycopeptides extracted from the ghosts of a patient firstly considered as affected by acquired dyserythropoietic anemia, but then resulting in a transient stage of erythroblastic hyperplasia during the recovery process of bone marrow aplasia. In the first of the two cases of congenital dyserythropoietic anemia type II (No. 1) sialic acid and galactosamine are present only in traces in ghost glycopeptides, and in the second case (No. 2) the content of these two components is also extremely low. Protein, hexose, and fucose content are within normal values.

Table II Glycopeptides isolated from erythrocyte ghosts of patients suffering from PNH polycythemia vera and unknown hemolytic anemia

	Normal values		PNH		Polycythemia vera	Hemolytic anemia and muscular dystrophy	
	young ghosts	old ghosts	7	8	9	10	11
Sialic acid	13.7 ± 1.2	9.9 ± 2.1	4.0	traces	12.3	traces	traces
Galactosamine	5.1 ± 0.1	4.4 ± 0.1	0.8	traces	5.6	3.0	1.0
Glucosamine	13.2 ± 1.9	14.0 ± 2.4	17.0	9.5	11.4	22.7	23.4
Fucose	4.1 ± 0.1	3.0 ± 0.1	ND	ND	2.4	5.2	4.2
Hexose	23.2 ± 0.1	23.2 ± 2.2	ND	ND	20.4	24.0	22.0
Protein	24.6 ± 2.2	25.5 ± 2.0	ND	ND	30.4	20.0	14.0

Values are expressed as g/100 g dry weight.

Paroxysmal nocturnal hemoglobinuria. Glycopeptides extracted from the PNH erythrocyte ghosts show an extremely low content of sialic acid and galactosamine (table II). Particularly in case No. 8, these two components are not detectable.

Hemolytic anemia with muscular dystrophy. As reported above two brothers were affected by muscular dystrophy associated with a serious form of hemolytic disorder which could not be framed in any of the known hemolytic diseases. Table II shows that in both cases sialic acid is not detectable in ghost glycopeptides. The galactosamine content is also very low, while glucosamine in concentration is higher than normal. Protein, fucose and hexose were within normal values.

Polycythemia vera. In the only case of polycythemia vera examined (table II) no variation with respect to the normal values was detected in ghost glycopeptides except a significantly higher protein content.

Discussion

Mechanisms by which erythrocytes in normal and pathological conditions are removed from the circulating blood are only partially known. Membrane structure certainly plays a fundamental role in the determination of red cell life span. In particular it was recently pointed out that sial

12 ROSENBERG, S A and GUIDOTTI, G The protein of human erythrocytes Archs Biochem Biophys *100* 119–130 (1963)
13 SCOTT, J E Aliphatic ammonium salts in the assay of acid polysaccharides from tissues Meth biochem Analysis *8* 145–197 (1960)
14 SPRINGER, G F Enzymatic and nonenzymatic alterations of erythrocyte surface antigens Bact Rev 27 191–227 (1963)
15 SVENNERHOLM, L. Quantiative estimation of sialic acid III An anion exchange resin method Acta chem scand *12* 547–554 (1958)
16 TREVELYAN, W E and HARRISON, J F Studies on yeast metabolism Fractionation and microdetermination of cell carbohydrates Biochem J *50* 298–303 (1952)
17 WALLACH, D F The plasma membrane Dynamic prospectives, genetics and pathology (Heidelberg Science Library, New York 1972)
18 WESEMAN, W and ZILLIKEN, F Receptors of neurotransmitters II Sialic acid metabolism and the serotonin induced contraction of smooth muscle Biochem Pharmac *16* 1773–1779 (1967)
19 WINZLER, R J Carbohydrates in cell surfaces Int Rev Cytol *29* 77–125 (1970)
20 YACHNIN, S, LAFORET, M T, and GARDNER, F H pH dependent hemolitic system I Their relationship to paroxysmal nocturnal hemoglobinuria Blood *17* 83–96 (1961)

Prof CESARE BALDUINI, Istituto di Chimica Biologica, Facoltà di Scienza, Piazza Botta, *I-27100 Pavia* (Italy)

Acta haemat. 55: 289-295 (1976)

Stimulation of Phagocytosis by Human Lysozyme

MATTI KLOCKARS and PETER ROBERTS

Fourth Department of Medicine, University Central Hospital, Helsinki

Key Words. Lysozyme · Neutrophil leukocytes · Phagocytosis

Abstract. Human lysozyme in physiologic concentrations (10-400 μg/ml) significantly ($p<0.001$) stimulates the phagocytosis of yeast cells by human polymorphonuclear leukocytes. This stimulating effect was observed even in the absence of serum factors. Hen egg white lysozyme or protamine sulfate had no effect on phagocytosis. The stimulating effect of human lysozyme was not the result of opsonization but was apparently related to the effects on constituent membranes of the phagocytic cells.

The presence of lysozyme (LZM) in phagocytic cells, particularly polymorphonuclear neutrophils (PMN), monocytes and macrophages, has been well documented [4, 5]. LZM has generally been considered to participate in both intracellular and extracellular bactericidal processes, primarily in the later stages of the phagocytic process. Recent observations on the effects of LZM on mammalian cells and their cytoplasmic membranes [11] suggest the possibility that LZM might also participate in the earlier stages of phagocytosis in which major membrane activities are involved [13].

To gain further insight into this possible role of LZM, we have studied the effect of homologous human LZM on the *in vitro* phagocytosis of yeast cells by human PMN.

Material and Methods

Phagocytosis. Buffy coat leukocytes were prepared by sedimentation from heparinized peripheral venous blood of healthy laboratory personnel. PMN were suspend-

ed in autologous plasma (heparin) or in various media in a concentration of 5 000 cells/μl Phagocytic activity was determined by the method of BRANDT [2] using heat killed Baker s yeast cells cells in suspensions with a PMN to yeast cell ratio of 1 10 After incubation for 30 min at 37 °C, the suspension was centrifuged and the resulting pellet was smeared on glass slides and stained with May Grunwald Giemsa 100 PMN were examined, and the mean number of yeast cells ingested per PMN was calculated and expressed as the 'phagocytic index' (PI)

Lysozyme Human LZM was isolated from the urine of patients with monocytic or myelomonocytic leukemia by the bentonite adsorption method [12] followed by ion exchange chromatography on CM 32 resin (Whatman) with a linear elution gradient of 0 05–0 5 M $NaHCO_3$, pH 8 0 [3] Hen egg white LZM (crystallized dialyzed and lyophilized three times) and protamine sulfate (from herring, grade III) were purchased from Sigma Chemical Co

Removal of LZM from serum was done with bentonite adsorption The bentonite was first washed with large quantities of Dulbecco s phosphate buffered saline, pH 7 2, and then added to serum at a concentration of 10 mg/ml The suspension was thoroughly mixed and incubated at room temperature for 30 min The LZM concentration of the serum supernatant after centrifugation, as measured by the lysoplate method [12] was zero

Opsonization To test the opsonizing effect of human LZM, the yeast cells were preincubated with LZM, 400 μg/ml, for 30 min at room temperature, washed with PBS and tested for susceptibility to phagocytosis

Gel electrophoresis Polyacrylamide gel electrophoresis in 8% polyacrylamide containing 0 1% SDS was performed according to LAEMMLI [9]

Results

The purity of human LZM was verified by polyacrylamide gel electrophoresis which demonstrated a single band (fig 1)

The PI of PMN obtained from 19 healthy persons was 4 6±0 3 (fig 2) When human LZM in concentrations of 10–400 μg/ml was added to the PMN suspended, as above, in heparinized autologous plasma, there was a significant increase in the ingestion of yeast cells The addition of 20 μg/ml of human LZM, for example, raised the PI to 5 4±0 4 ($p<0$ 001) In contrast to the effect of human LZM, neither protamine sulfate nor hen egg white LZM stimulated phagocytosis No morphological changes in the PMN were visible by light microscopy, and no effect on the viability of the PMN in the presence of up to 400 μg/ml of LZM could be demonstrated by trypan blue dye exclusion

To determine the PI of PMN incubated in LZM-free medium (except for the endogenous LZM of PMN), the cells were washed three times with Medium 199 and suspended in autologous normal serum and in au-

Fig. 1. Polyacrylamide gel electrophoresis of 25 μg of human LZM (A) isolated by bentonite adsorption and chromatography on carboxymethyl cellulose (CM 32 resin) and hen egg white LZM (B) in 8% polyacrylamide slab gels containing 0.1% SDS according to LAEMMLI [9].

tologous serum from which LZM had been removed by bentonite adsorption (table I). The PI of PMN suspended in normal autologous serum was 5.1 ± 0.3. When the serum had been adsorbed with bentonite, the PI decreased to 4.5 ± 0.3 ($p<0.001$). The PI of PMN suspended in serum free or plasma free medium was reduced to 0.5 ± 0.2, but even in the absence of serum or plasma, human LZM stimulated phagocytosis to a PI of 0.9 ± 0.2 ($p<0.001$). A comparable decrease in the phagocytic activity of PMN was observed when the cells were suspended in heat inactivated serum. Even under these conditions, LZM apparently stimulated the phagocytic activity of PMN, although not to a statistically significant level.

When the PMN from 9 persons were exposed to untreated or LZM treated (opsonized) yeast cells, the PI was 4.7 ± 0.4 and 4.8 ± 0.3, respectively (not statistically significant).

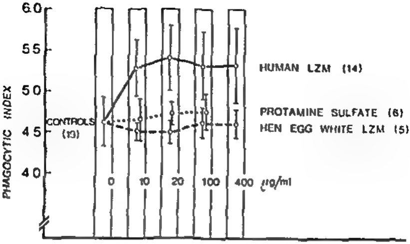


Fig 2 PI (mean ± SD) of human PMN in the presence of 10–400 µg/ml of human LZM, hen egg white LZM and protamine sulfate In parentheses are the numbers of individuals tested for each agent

Table I Effect of LZM on the phagocytosis of yeast cells by human PMN

Medium[1]	Phagocytic index mean±SD	Number of individuals tested	p value[2]
Autologous serum	5 1±0 3	16	<0 001
Bentonite-adsorbed autologous serum	4 5±0 3	13	
Medium 199	0 5±0 2	10	<0 001
Medium 199 + human LZM, 100 µg/ml	0 9±0 2	10	
Heat inactivated autologous serum, +56°C for 15 min	0 5±0 3	3	not significant
Heat inactivated autologous serum + human LZM, 100 µg/ml	0 6±0 3	3	

[1] Cells were washed three times in Medium 199 and thereafter suspended in the various media

[2] Student's t test

Discussion

The initial steps in the phagocytic process involve the attachment of the material to be ingested to the external surface of the cell, the formation of pseudopodia and the phagocytic vesicle [13] Whether a microor-

ganism is engulfed depends on the interaction of several factors including opsonins (antibodies) complement and other heat labile factors and the cell membrane of the phagocyte [14] Previous studies [7] have suggested that LZM might act in certain circumstances as an opsonin in the phagocytosis of bacteria independently of the LZM sensitivity of the bacteria LYCKE *et al* [10] and SENECA and PEER [15] have also described an enhancing effect of high concentrations (1 000 μg/ml) of hen egg white LZM on the phagocytosis of *Toxoplasma gondii* by HeLa cells and of *Proteus vulgaris* and *Streptococcus haemolyticus* by human PMN With respect to the present studies it is particularly noteworthy that the stimulation of phagocytosis was demonstrated only with the homologous human LZM and that the concentrations used are probably within the range achieved in inflammatory exudates *in vivo* [16]

Time sequence studies on the degranulation of PMN that accompanies phagocytosis have shown that the initial events are related to changes in those specific granules that contain most of the LZM in the PMN [6 17] LZM is released into the extracellular medium early during phagocytosis without any accompanying appreciable rise in enzyme activity in the cytoplasm or any indication of cellular damage We might therefore, postulate that this release of LZM affects a local milieu of autostimulation of phagocytosis in the very early stages of the phagocytic process

The present studies indicate that LZM does not act as a true opsonin since its action is on the phagocyte and not the target organism Although LZM stimulated phagocytosis in serum free media the significance of serum factors in the engulfment process was apparent Whether the decrease in PI of PMN suspended in heparinized plasma as compared with cells suspended in serum was related to the 30–40% inhibition of LZM activity caused by heparin needs further investigation [5 and unpublished observations]

The cationic LZM molecule in addition to causing structural changes in certain cell membranes [11] could possibly also function as 'glue' in the process of particle attachment Whereas adhesion *per se* of particles to the PMN surface does not produce maximal ingestion such adhesion probably promotes ingestion both in the absence or presence of serum factors When serum IgG participates it appears that besides promoting or stimulating particle attachment it also triggers the ingestion step [14]

Thus far no specific substrate for LZM in mammalian cells has been identified The stimulating effect of species-specific LZM on the phagocytic activity of PMN could not be achieved with LZM isolated from hen

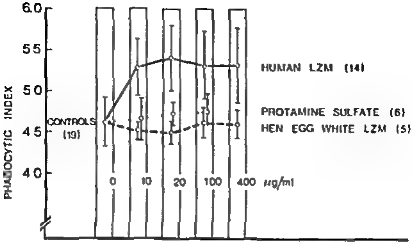


Fig. 2. PI (mean ± SD) of human PMN in the presence of 10–400 μg/ml of human LZM, hen egg white LZM and protamine sulfate. In parentheses are the numbers of individuals tested for each agent.

Table I. Effect of LZM on the phagocytosis of yeast cells by human PMN

Medium[1]	Phagocytic index mean±SD	Number of individuals tested	p value[2]
Autologous serum	5.1±0.3	16	<0.001
Bentonite adsorbed autologous serum	4.5±0.3	13	
Medium 199	0.5±0.2	10	<0.001
Medium 199+human LZM 100 μg/ml	0.9±0.2	10	
Heat inactivated autologous serum +56°C for 15 min	0.5±0.3	3	not significant
Heat inactivated autologous serum + human LZM 100 μg/ml	0.6±0.3	3	

[1] Cells were washed three times in Medium 199 and thereafter suspended in the various media.
[2] Student's t test.

Discussion

The initial steps in the phagocytic process involve the attachment of the material to be ingested to the external surface of the cell, the formation of pseudopodia and the phagocytic vesicle [13]. Whether a microor-

ganism is engulfed depends on the interaction of several factors including opsonins (antibodies) complement and other heat labile factors and the cell membrane of the phagocyte [14] Previous studies [7] have suggested that LZM might act in certain circumstances as an opsonin in the phagocytosis of bacteria independently of the LZM sensitivity of the bacteria LYCKE *et al* [10] and SENECA and PEER [15] have also described an enhancing effect of high concentrations (1 000 μg ml) of hen egg white LZM on the phagocytosis of *Toxoplasma gondii* by HeLa cells, and of *Proteus vulgaris* and *Streptococcus haemolyticus* by human PMN With respect to the present studies it is particularly noteworthy that the stimulation of phagocytosis was demonstrated only with the homologous human LZM and that the concentrations used are probably within the range achieved in inflammatory exudates *in vivo* [16]

Time sequence studies on the degranulation of PMN that accompanies phagocytosis have shown that the initial events are related to changes in those specific granules that contain most of the LZM in the PMN [6 17] LZM is released into the extracellular medium early during phagocytosis without any accompanying appreciable rise in enzyme activity in the cytoplasm or any indication of cellular damage We might, therefore postulate that this release of LZM affects a local milieu of 'autostimulation' of phagocytosis in the very early stages of the phagocytic process

The present studies indicate that LZM does not act as a true opsonin since its action is on the phagocyte and not the target organism Although LZM stimulated phagocytosis in serum free media the significance of serum factors in the engulfment process was apparent Whether the decrease in PI of PMN suspended in heparinized plasma as compared with cells suspended in serum was related to the 30–40% inhibition of LZM activity caused by heparin needs further investigation [8 and unpublished observations]

The cationic LZM molecule in addition to causing structural changes in certain cell membranes [11] could possibly also function as glue in the process of particle attachment Whereas adhesion *per se* of particles to the PMN surface does not produce maximal ingestion such adhesion probably promotes ingestion both in the absence or presence of serum factors. When serum IgG participates it appears that besides promoting or stimulating particle attachment it also triggers the ingestion step [14]

Thus far no specific substrate for LZM in mammalian cells has been identified The stimulating effect of species-specific LZM on the phagocytic activity of PMN could not be achieved with LZM isolated from hen

egg white, and presently available evidence indicates that hen and human LZM have the same substrate specificity and cationic properties There are, however, significant differences in the primary and tertiary structures of these two enzyme [1], and these may be involved in the stimulating effect of human LZM on phagocytosis

Acknowledgements We thank Dr ELLIOTT F OSSERMAN for providing us with human lysozyme, Dr C G GAHMBERG for the gel electrophoresis studies and Ms LEENA JUUSELA and Ms TUULA VESTERINEN for excellent technical assistance

This work was supported by grants from The Finnish National Research Council for Medical Sciences and The Sigrid Jusélius Foundation

References

1 BLAKE, C C F and SWAN, I D A X ray analysis of structure of human lysozyme at 6 Ångström resolution Nature new Biol *232* 12–15 (1971)
2 BRANDT, L Studies on the phagocytic activity of neutrophilic leucocytes Scand *J Haematol*, suppl *2* (1967)
3 CANFIELD R E and ANFINSEN, C B Nonuniform labelling of egg white lysozyme Biochemistry, N Y *2* 1073–1078 (1963)
4 COHN, Z A and HIRSCH, J G The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leukocytes J exp Med *112* 983–1004 (1960)
5 COHN, Z A and WIENER, L The particulate hydrolases of macrophages I Comparative enzymology, isolation and properties J exp Med *118* 991–1009 (1963)
6 ESTENSEN, R D, WHITE, J G, and HOLMES B Specific degranulation of human polymorphonuclear leukocytes Nature, Lond *248* 347–348 (1974)
7 GLADSTONE, G P and JOHNSTON, H H The effect of cultural conditions on susceptibility of *Bacillus anthracis* to lysozyme Br J exp Path *36* 363–372 (1955)
8 KERBY, G P and EADIE G S Inhibition of lysozyme by heparin Proc Soc exp Biol Med *83* 111–113 (1953)
9 LAEMMLI, U K Cleavage of structural proteins during the assembly of the head of bacteriophage T 4 Nature, Lond *227* 680 685 (1970)
10 LYCKE, E, LUND E, and STRANNEGÅRD O Enhancement by lysozyme and hyaluronidase of the penetration by *Toxoplasma gondii* into cultured host cells Br J exp Path *46* 189–199 (1965)
11 OSSERMAN E F KLOCKARS M, HALPER, J, and FISCHEL, R E Effects of lysozyme on normal and transformed mammalian cells Nature, Lond *243* 331–335 (1973)
12 OSSERMAN F F and LAWLOR D P Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia J exp Med *124* 921–952 (1966)
…AL, P PINTO, J, and HANIFIN, J M Surface morphology of human

mononuclear phagocytes during maturation and phagocytosis. J. Ultrastruct. Res. 45: 216–226 (1974).

14 RABINOVITCH, M.: Phagocytosis: the engulfment stage. Seminars Hematol. 5: 134–155 (1968).

15 SENECA, H. and PEER, P.: Effect of antibacterials, antibiotics, enzymes and steroids on phagocytosis. J. Am. Geriat. Soc. 14: 187–199 (1966).

16 SENN, H. J.; CHU, B.; O'MALLEY, J., and HOLLAND, J. F.: Experimental and clinical studies on muramidase (lysozyme). I. Muramidase activity of normal human blood cells and inflammatory exudates. Acta haemat. 44: 65–77 (1970).

17 WRIGHT, D. G. and MALAWISTA, S. E.: The mobilization and extracellular release of granular enzymes from human leukocytes during phagocytosis. J. Cell Biol. 53: 788–797 (1972).

Dr. M. KLOCKARS, 4th Department of Medicine, Unioninkatu 38, SF-00170 Helsinki 17 (Finland)

Acta haemat 55 296-299 (1976)

Salicylamide-Glucuronide Formation in Children with Favism and in Their Parents

STEFANO CUTILLO, SINFAROSA COSTA, MARIA CRISTINA VINTULEDDU and TULLIO MELONI

University Children Hospital, Sassari

Key Words Favism · G-6-PD deficiency · Liver function in favism · Salicylamide-glucuronide

Abstract Salicylamide glucuronide formation has been determined in 27 children who underwent a favism crisis, 25 parents, and in 25 normal children who served as controls. A highly significant mean lower glucuronide formation was observed in the favism group in respect to the controls. The difference between fabic children and their parents was significant, and between parents and controls there was no significant difference.

A deficiency in the G-6-PD activity of mature red cells leads to hemolytic crises after ingestion of fava beans, administration of some drugs or, more rarely, in the course of viral hepatitis. An increased red cell turnover [1] or severe hemolysis [2] have been reported in isolated cases of hyperbilirubinemia in newborns with such a deficiency, but research we performed on a large casuistry of mature newborns, demonstrated that this form of severe neonatal jaundice is 'paradoxically' not hemolytic in origin. We postulated a possible liver involvement associated with G-6-PD deficiency which deteriorates the postnatal transiently impaired liver ability to metabolize bilirubin [3]. The idea that G-6-PD deficiency could be considered as a cause of metabolic impairment other than in erythrocytes received experimental support, as it was demonstrated that salicylamide-glucuronide formation is significantly decreased in enzyme-deficient hyperbilirubinemic newborns [4] and in children who experienced a favism crisis [5].

In the present paper the problem of salicylamide-glucuronide formation has been reexamined in a group of children with favism and in their parents.

Materials and Methods

Salicylamide glucuronide formation was determined in 27 children (13 males and 14 females, 7 of whom were heterozygotes) whose age ranged between 2 and 9 years, admitted to the clinic after a hemolytic crisis due to fava bean ingestion. Hemoglobinuria occurred in all cases and anemia was more or less pronounced (Hb range 5–8.2 g%). The latter was corrected within 24–72 h from admittance by two or more blood transfusions. Salicylamide was administered 2 or 3 days after the last transfusion and the Hb level was in no case less than 10.5 g%. The test was also carried out on some volunteers among the parents of the affected children: 8 affected fathers of females and 17 mothers (10 of whom were homo- and 7 heterozygotes). 25 healthy children without hematological traits aged 6–12 years served as controls. Diagnosis of G-6-PD deficiency was made in all cases, using the cytochemical test of SANSONE *et al.* [6], on venous blood which was taken from the affected children immediately before blood transfusion. The salicylamide (Urtosal Heumann) test was performed by giving a single 20 mg/kg body weight oral dose of salicylamide diluted in water in the morning after breakfast, and urine was collected during the next 24 h. Total salicylamide and salicylamide-glucuronide were determined by the method of LEVY and MATSUZAWA [7].

Results

The mean salicylamide-glucuronide formation was 31.25 ± 20.49% in the fabic children, 49.59 ± 19.94% in their parents and 56.18 ± 11.30% in the control subjects. The difference between fabic and normal children was highly significant ($p < 0.001$). No significance was found between parents and normal children ($p > 0.1$) and little significance between fabic children and their parents ($p < 0.01$).

Discussion

The data show that in children with favism the mean glucuronide formation is significantly lower than in normal subjects, thus confirming the results of CASSIMOS *et al.* [5] who found that glucuronide formation was less than 23% (arbitrarily established as the borderline between normal and affected persons) in 12 of 18 cases. In our favism group in 16 of 27 (59%) the glucuronide formation was lower than 33% (the minimum value found in the normal subjects). The mean values of the parents were not statistically different from the controls, however, in 7 of 25 (28%) of the former the values were lower than 33% and 5 of these had experienced a favism crisis at some time or other. It is noteworthy that no difference was observed be-

Table 1 Salicylamide-glucoronide excretion in urine (% of total) in children who experienced a favism crisis, in a group of their parents, and in controls

Children with favism			Parents			Controls		
No	sex	urine excretion	No	sex	urine excretion	No	sex	urine excretion
1	ma	22 2	1	fe ho	54 8	1	ma	41 6
2	fe he	17 0	2	fe he	45 2	2	ma	47 0
3	fe ho	63 2	3	fe he	70 7	3	ma	61 5
4	fe he	18 4	4	ma	87 6	4	fe	55 5
5	fe-ho	29 2	5	fe-he	71 5	5	fe	45 2
6	ma	41 1	6	fe he	20 8	6	fe	61 4
7	fe-he	10 8	7	fe ho	56 7	7	fe	51 5
8	ma	54 8	8	ma	33 3	8	ma	68 7
9	fe he	75 4	9	ma	33 3	9	fe	80 2
10	fe-ho	6 6	10	fe ho	25 8	10	ma	49 0
11	fe-he	6 6	11	fe ho	38 8	11	ma	66 2
12	ma	39 0	12	ma	55 4	12	ma	33 4
13	ma	42.0	13	fe ho	79 2	13	ma	61 6
14	fe ho	5 9	14	ma	32.1	14	ma	60 0
15	ma	32 7	15	ma	76 3	15	fe	70 3
16	fe ho	8 8	16	fe-he	46 4	16	fe	67 8
17	fe he	44 5	17	ma	48 3	17	ma	53 3
18	ma	32 0	18	fe ho	62 5	18	ma	60 4
19	fe ho	57 3	19	fe-ho	28 4	19	fe	41 8
20	ma	68 8	20	ma	41 6	20	fe	63 1
21	ma	34 2	21	fe ho	66 4	21	ma	53 1
22	fe ho	19 0	22	fe ho	8 2	22	ma	57 1
23	fe he	50 0	23	fe ho	64 4	23	ma	36 8
24	ma	8 6	24	fe he	53 3	24	ma	66 0
25	ma	28 5	25	fe he	36 7	25	ma	51 1
26	ma	16 2						
27	ma	12 2						
Mean		31 35			49 59			56 18
SD		20 49			19 94			11 30

ma = Male, fe ho = female homozygous fe he = female heterozygous

tween the homo and heterozygotes with respect to glucuronide activity Furthermore, it should be stressed that low glucuronide activity was paralleled between affected children and their parents only in 4 cases 1 male, 1 heterozygous and 2 homozygous females No such parallelism was found in the remainder

An overall consideration of these data points to a probable link which has not yet been clarified between G-6-PD deficiency and liver ability in glucuronizing salicylamide. What relationship, if any, this defect may bear to favism crises is only a matter of speculation, whereas it seems possible that there is a connection between this impaired liver ability as demonstrated in the neonatal age too [4, 8] and hyperbilirubinemia in mature newborns.

References

1 VALAES, T., KARAKLIS, A., STRAVRAKAKIS, D., BAVELA-STRAVRAKAKIS, K., PERAKIS, A., and DOXIADIS, S. A.: Incidence and mechanism of neonatal jaundice related to glucose-6-phosphate dehydrogenase deficiency. Pediat. Res. *3:* 448 (1969).

2 LOPEZ, R. and COOPERMAN, J. M.: Glucose-6-phosphate dehydrogenase deficiency and hyperbilirubinemia in the newborn. Am. J. Dis. Child. *122:* 66 (1971).

3 MELONI, T., CAGNAZZO, G., DORE, A., and CUTILLO, S.: Phenobarbital for prevention of hyperbilirubinemia in glucose-6-phosphate dehydrogenase-deficient newborn infants. J. Pediat. *82:* 1048 (1973).

4 MALAKA-ZAFIRIU, K., TSIURES, T., DANIELIDES, B., and CASSIMOS, C.: Salicylamide glucuronide formation in newborns with severe jaundice of unknown etiology and due to glucose-6-phosphate dehydrogenase deficiency in Greece. Helv. paediat. Acta *28:* 323 (1973).

5 CASSIMOS, C., MALAKA-ZAFIRIU, K., TSIURES, T., and DANIELIDES, B.: Variations in salicylamide glucuronide formation in normal and in G-6-PD-deficient children. J. Pediat. *85:* 110 (1974).

6 SANSONE, G., RASORE-QUARTINO, A. e VENEZIANO, G.: Dimostrazione su strisci di sangue di una doppia popolazione eritrocitaria nelle donne eterozigoti per la deficienza in glucoso-6-P-deidrogenasi. Pathologica *55:* 371 (1963).

7 LEVY, G. and MATSUZAWA, T.: Pharmacokinetics of salicylamide elimination in man. J. Pharmac. exp. Ther. *156:* 285 (1967).

8 MELONI, T., COSTA, S., CORTI, R., and CUTILLO, S.: Salicylamide glucuronide formation in newborn babies with G-6-PD deficiency (in press).

Prof. S. CUTILLO, Clinica Pediatrica, Viale S. Pietro 12, *I-07100 Sassari* (Italy)

Acta haemat 55 300-305 (1976)

Blood Group Phenotypes and Hemoglobin S

An Anthropologic Study in Two Israeli Arab Communities

CYRIL LEVENE, ELIEZER A RACHMILEWITZ, ERIC EZEKIEL, EMIL FREUNDLICH and GERALD SANDLER

Reference Laboratory of Immunohematology and Blood Groups, Government Central Laboratories, Ministry of Health, Department of Hematology, Hadassah University Hospital and Hebrew University Hadassah Medical School, Blood Bank, Hadassah University Hospital, Jerusalem, and Departments of Hematology and Pediatrics, Nahariya Government Hospital Nahariya

Key Words Anthropology Blood groups Hemoglobin S Israeli Arabs

Abstract Blood group phenotypes of anthropologic significance are described for inhabitants of two Israeli Arab communities with foci of hemoglobin S (HbS) The presence of Fy(a-b-) Rh_0 hr^{V+}, and Js(a+) among the Hulah Valley Bedouin and of *Fy(a-b-), Rh_0 and hr^{V+} in Acre are indicative of genetic admixture of* African origin These non African foci of HbS are thereby distinguished from previously described HbS foci in India Greece, and Turkey where low or absent Rh_0 phenotypes imply secondary dispersions of the HbS gene from the Arabian peninsula

The present distribution of sickle cell hemoglobin (HbS) in non-black populations of Europe, Asia, and the Americas is attributable either to *multiple mutations at the HbS locus, or more plausibly, to a single muta-*tion whose dispersion reflects historical population migrations [7] Genetic and historic evidence favor an equatorial African origin for the HbS mutation which diffused east to Arabia, and subsequently to Europe and Asia [8] An alternative hypothesis, which emphasized the relatively low frequency of the African blood group phenotype Rh_0 (cDe) in HbS foci of Greece, Turkey, and India, proposes that the HbS mutant arose in Saudi Arabia, spreading west to Africa, north to Europe, and east to India [12] In either instance, HbS among non-black populations of Europe and Asia appears to represent secondary dispersions from Arabia, and the persistance of HbS in high concentrations in these isolated foci probably

reflects local selective factors including endemic malaria [16] and G6PD deficiency [14].

The following report describes the blood group anthropology of two communities of Israeli Arabs with HbS. In contrast to previously described studies of HbS among European and Asian populations, the present report documents a remarkably high correlation of HbS with specific blood group phenotypes, indicating the genetic individuality of non-African foci of HbS.

Materials and Methods

Hulah Valley Bedouin. In the first of two population surveys, specimens were obtained from 132 Bedouin representing 17 Arab families who reside in the Hulah Valley, north of the Sea of Galilee. The region was endemic for *P. falciparum* and *P. vivax* until the mid 1930s. The origin of the Hulah Valley Bedouin is obscure, but it is most probable that their ancestors migrated several hundred years ago from the area that is presently identified as northern Iraq. In physical appearance, the Hulah Valley Bedouin are not overtly Negroid and are indistinguishable from Arabs of neighboring villages. Sickle cell disease in these Bedouin was first described in 1955 [19].

Acre. The second population survey consisted of specimens from 32 urban Arabs, representing 8 families from the coastal city of Acre. Sickle cell disease in this community was first detected in 1973 and the clinical details as well as those for the Hulah Valley Bedouin, have been described elsewhere [22].

The propositi, two children with sickle cell disease (HbSS), were not obviously Negroid in physical appearance. However, in the course of the field study, and with the knowledge that HbS was present in the community, the authors did note Negroid features among other inhabitants of the community.

Blood group and hemoglobin typing. In addition to routine ABO and Rh blood group phenotyping, fresh specimens were tested for the following antigens: M, N, S, s, Fy^a, Fy^b, Js^a and hr^V (V). The red cell phenotypes Fy(a− b−), Rh_0 (cDe), Js(a+) and $hr^{V}+$ are individually almost diagnostic of African origin [4, 21]. Combinations of these antigens without evidence of African ancestry have not been described. Fy(a− b−), probably the most reliable African blood group phenotype, was not present among 9,167 Caucasians [13], but has been detected in 80–97% of West Africans [21].

Rh_0, occurring in 2–3% of Caucasians, is found in 50–90% of African tribesmen [20]. The antigens hr^V (V) and Js(a+) occur in less than 1% of Caucasians, but in 47% [5] and 27% [9], respectively, of West Africans. There are no published data for the frequencies of phenotypes Fy(a− b−), Rh_0 and Js(a+) for the indigenous Arab populations of Israel. However, the presence of phenotypes Rh_0, Fy(a− b−) and hr^V in South Sinai Bedouin [3], Rh_0 and hr^V+ in Saudi Arabia [15] and Rh_0 in Sephardi [17], North African [10] and Arabian Jews [2] are all considered to reflect African admixture.

Table I Relationship of specific blood group phenotypes and hemoglobin types in two communities with foci of HbS

Blood group Phenotype	Fy (a–b–)	Rh_o	Js (a+)	hr^{V+}	Fy (a–b–) Rh_o	Fy (a–b–) Js (a+)	Fy (a–b–) hr^{V+}	Rh_o hr^{V+}	Fy (a–b–) Rh_o hr^{V+}	Fy (a–b–) Rh_o Js (a+)	None	Total
Hulah Valley												
HbSS	5	1	1	0	0	1	0	0	0	0	3	11
HbAS	22	0	2	0	2	5	1	1	0	1	6	40
HbAA	36	0	0	0	4	4	1	0	0	0	36	81
Acre												
HbSS	2	0	0	0	1	0	0	0	2	0	0	5
HbAS	9	1	0	0	2	0	1	0	3	0	1	17
HbAA	2	0	0	1	1	0	0	0	1	0	1	6
HbCC	1	0	0	0	0	0	0	0	0	0	0	1
HbAC	3	0	0	0	0	0	0	0	0	0	0	3

Table II Distribution and frequencies of selected blood group phenotypes

Phenotype	West African[1] blacks, %	Caucasians[1] %	Hulah Valley %	Hulah Valley number	Acre %	Acre number
Fy (a- b-)	90	0	62	[illegible]	88	28
Rh_0	60	3	7	9	[illegible]	12
Js (a+)	[illegible]	0	11	14	0	0
hr^{V+}	40	0.5	2	3	25	8

[1] Data summarized from previous surveys [4, 21].

At the time of testing, the only anti hr^{V+} reagent available was specific for type O red cells and, therefore, the hr^{V+} results reflect testing of only type O specimens.

Hemoglobin types were determined by starch gel electrophoresis and in homozygotes, by the appearance of sickle cells in fresh peripheral blood smears. Hemoglobin typings were confirmed in the laboratory of Dr. T. H. Huisman, Atlanta, Ga.

Results

Hulah Valley. The hemoglobin types of 132 Hulah Valley Bedouin (representing 17 families) were HbSS (11), HbAS (40) and HbAA (81) (table I). African blood group markers were prevalent. 66% of the sampling had at least one of the specific blood group phenotypes and 16% had two or more. 62% of the sampling were Fy(a-b-), 7% were Rh_0, 11% were Js(a+), and 2% were hr^{V+} (table II).

Acre. The hemoglobin types of 32 Acre Arabs (representing 8 families) were HbSS (5), HbAS (17), HbAA (6), HbCC (1), and HbAC (3) (table I). In this sampling 94% had at least one of the specific blood group phenotypes and 38% had two or more. Although the phenotype Js(a+) was not present, the other specific blood group phenotypes were more prevalent than in the Hulah Valley (table II). 85% were Fy(a-b-), 38% were Rh_0, and 25% hr^{V+}.

Discussion

The present known distribution of HbS in non blacks includes inhabitants of Greece, Sardinia, Italy, Turkey, Libya, Tunisia, Iran, Saudi Arabia, India and Southeast Asia [1]. HbS has not been found in Israeli

Jews [24], but has been described previously in Israeli Arabs [19]. HbS has been reported in the United States in Caucasians with no apparent black ancestry [11], where the incidence may be as high as 0.08% [18].

The present report illustrates the role of blood group anthropology in the study of HbS in populations and in individuals without physical or known historical evidence of African ancestry. The presence of phenotypes Fy(a–b–), Rh_o, Js(a+) and hr^{V+} in the Hulah Valley Bedouin and Fy(a–b–), Rh_o and Js(a+) in Acre, documents direct African admixture. The fortuitous finding of HbCC and AC in Acre is additional evidence of genetic admixture from West Africa, since HbC is highly localized to West Africa [6] and is not found elsewhere without evidence of African ancestry. The four individuals with HbC in the present study are relatives of a large family of Bedouin with a known high incidence of HbC [23].

Acknowledgements. The authors express their appreciation to R. SELA and Y. RUDOLPHSON for technical assistance, to H. KANAANEH of the Ministry of Health, Acre, for invaluable work in preparing the family trees, to S. JACOBSON for preparation of manuscript, and to MARY WALKER of the New York Blood Center, and to ALLEN GELB for providing rare antisera.

Grant support: Benson Rosenberg Research Funds, Israel Council for Research and Development, and WHO.

References

1 BERNSTEIN, R. E.: Mass screens for sickle cell disease. J. Am. med. Ass. *223*: 438 (1973).

2 BONNÉ, B.; ASHBEL, S.; MODAI, M.; GODBER, M. J.; MOURANT, A. E.; TILLS, D., and WOODHEAD, B. J.: The Habbanite isolate. I. Genetic markers in the blood. Hum. Hered. *20*: 609–622 (1970).

3 BONNÉ, B.; GODBER, M.; ASHBEL, S.; MOURANT, A. E., and TILLS, D.: South Sinai Bedouin. Preliminary report on their inherited blood factors. Am. J. phys. Anthrop. *34*: 397–407 (1971).

4 BOYD, W. C.: Genetics and the human race. Science, N.Y. *140*: 1057–1064 (1963).

5 NATALE, A. DE; CAHAN, A.; JACK, J. A.; RACE, R. R., and SANGER, R.: V, a 'new' Rh antigen, common in Negroes, rare in white people. J. Am. med. Ass. *159*: 247–250 (1955).

6 EDINGTON, G. M. and LEHMANN, H.: The distribution of hemoglobin C disease in West Africa. Man *36*: 1 (1956).

7 GELPI, A. P.: Migrant populations and the diffusion of the sickle cell gene. Ann. intern. Med. *79*: 258–264 (1973).

8 GELPI, A. P. and PERRINE, P.: Sickle cell disease and trait in white populations. J. Am. med. Ass. 224: 605–608 (1973)

9 GIBLETT, E. R.: Js, a 'new' blood group antigen found in Negroes. Nature, Lond. 181: 1221 (1958)

10 GUREVITCH, J., HASSON, E., MARGOLIS, E., and POLIAKOFF, C.: Blood groups in Jews from Tripolitania. Ann. hum. Genet. 19: 260–261 (1955)

11 KEELING, M. M., LOCKWOOD, W. B., and HARRIS, E. A.: Avascular necrosis and erythrocytosis in sickle-cell trait. New Engl. J. Med. 290: 442–444 (1974)

12 LEHMANN, H. and HUNTSMAN, R. G.: Man's haemoglobins, pp. 197–198 (Lippincott, Philadelphia 1966)

13 LEWIS, M., KAITA, H., and CHOWN, B.: The Duffy blood group system in Caucasians. A further population sample. Vox Sang. 23: 523–527 (1972)

14 LEWIS, R. A. and HATHORN, M.: Correlation of S hemoglobin with glucose-6-phosphate dehydrogenase deficiency and its significance. Blood 26: 176–179 (1965)

15 LISTER, R. W., ORR, N. W. M., BOTTING, D., IKIN, E. W., MOURANT, A. E., and LEHMANN, H.: The blood groups and haemoglobin of the Bedouin of Socotra. Man 1: 82–86 (1966)

16 LIVINGSTONE, F. B.: Malaria and human polymorphisms, in ROMAN, SANDLER and CAMPBELL, Annual review of genetics, pp. 33–64 (Annual Reviews, Palo Alto 1971)

17 MARGOLIS, E., GUREVITCH, J., and HERMONI, D.: Blood groups in Sephardic Jews. Am. J. phys. Anthrop. 18: 197–199 (1960)

18 MCGREW, C. J., jr: Sickle cell trait in the white population. J. Am. med. Ass. 224: 1762–1763 (1973)

19 MOTTS, W. S., MATHOTH, Y., and LEVIN, S.: Sickle cell disease in Israel. Report of 5 cases in an Arab family. Harefuah 51: 281 (1956)

20 MOURANT, A. E.: The distribution of the human blood groups, p. 438 (Blackwell, Oxford 1954)

21 RACE, R. R. and SANGER, R.: Blood groups in man, pp. 178–464 (Davis, Philadelphia 1968)

22 RACHMILEWITZ, E. A., LEVENE, C., HUTMAN, T. H. J., FREUNDLICH, E., and SANDLER, S. G.: Negro blood group markers and 'relatively benign' sickle cell disease in Israel. Abstract Proc. 1st nat. Sickle Cell Disease Conf., Bethesda 1974 (in press)

23 RACHMILEWITZ, E. A., LEVIN, S., and HUTMAN, T. H. J.: High frequency of hemoglobin C in an Israel Bedouin tribe. Israel J. med. Scis 10: 219–224 (1974)

24 RAMOT, B. and SHEBA, C. H.: The genetics of Israel's populations, in GOLDSCHMIDT Genetics of migrant and isolated populations, pp. 253–355 (Williams & Wilkins, Baltimore 1963)

CYRIL LEVENE, MD, Director, Reference Laboratory of Immunohematology and Blood Groups, Government Central Laboratories, Ministry of Health, 65 Yaffo Road, PO Box 6115, *Jerusalem* (Israel)

Jews [24], but has been described previously in Israeli Arabs [19]. HbS has been reported in the United States in Caucasians with no apparent black ancestry [11], where the incidence may be as high as 0.08% [18].

The present report illustrates the role of blood group anthropology in the study of HbS in populations and in individuals without physical or known historical evidence of African ancestry. The presence of phenotypes Fy(a–b–), Rh_o, Js(a+) and hrV+ in the Hulah Valley Bedouin and Fy(a–b–), Rh_o and Js(a+) in Acre, documents direct African admixture. The fortuitous finding of HbCC and AC in Acre is additional evidence of genetic admixture from West Africa, since HbC is highly localized to West Africa [6] and is not found elsewhere without evidence of African ancestry. The four individuals with HbC in the present study are relatives of a large family of Bedouin with a known high incidence of HbC [23].

Acknowledgements. The authors express their appreciation to R. SELA and Y. RUDOLPHSON for technical assistance, to H. KANAANEH of the Ministry of Health, Acre, for invaluable work in preparing the family trees, to S. JACOBSON for preparation of manuscript, and to MARY WALKER of the New York Blood Center, and to ALLEN GELB for providing rare antisera.

Grant support: Benson Rosenberg Research Funds, Israel Council for Research and Development, and WHO.

References

1 BERNSTEIN, R. E.: Mass screens for sickle cell disease. J. Am. med. Ass. *223:* 435 (1973).
2 BONNÉ, B.; ASHBEL, S.; MODAI, M.; GODBER, M. J.; MOURANT, A. E.; TILLS, D., and WOODHEAD, B. J.: The Habbanite isolate. I. Genetic markers in the blood. *Hum. Hered. 20:* 609–622 (1970).
3 BONNÉ, B.; GODBER, M.; ASHBEL, S.; MOURANT, A. E., and TILLS, D.: South Sinai Bedouin. Preliminary report on their inherited blood factors. Am. J. phys. Anthrop. *34:* 397–407 (1971).
4 BOYD, W. C.: Genetics and the human race. Science, N.Y. *140:* 1057–1064 (1963).
5 NATALE, A. DE; CAHAN, A.; JACK, J. A.; RACE, R. R., and SANGER, R.: V, a 'new' Rh antigen common in Negroes, rare in white people. J. Am. med. Ass. *159:* 247–250 (1955).
6 EDINGTON, G. M. and LEHMANN, H.: The distribution of hemoglobin C disease in West Africa. Man *36:* 1 (1956).
7 GELPI, A. P.: Migrant populations and the diffusion of the sickle-cell gene. Ann. intern. Med. *79:* 258–264 (1973).

8 GELPI, A. P and PERRINE, P., Sickle cell disease and trait in white populations. J Am. med Ass. 224 605–608 (1973)
9 GIBLETT E. R. Js, a 'new' blood group antigen found in Negroes. Nature, Lond. 181 1221 (1958)
10 GUREVITCH J, HASSON E., MARGOLIS E., and POLIAKOFF C. Blood groups in Jews from Tripolitania. Ann. hum. Genet. 19 260–261 (1955)
11 KEELING M M, LOCKWOOD W B., and HARRIS, E. A. Avascular necrosis and erythrocytosis in sickle-cell trait. New Engl J Med. 289 442–444 (1974)
12 LEHMANN H and HUNTSMAN, R. G., Man's haemoglobins, pp 197–198 (Lippincott, Philadelphia 1966)
13 LEWIS M, KAITA, H., and CHOWN, B The Duffy blood group system in Caucasians. A further population sample Vox Sang. 23 523–527 (1972)
14 LEWIS, R A and HATHORN M Correlation of S hemoglobin with glucose-6-phosphate dehydrogenase deficiency and its significance Blood 26 176–179 (1965)
15 LISTER, R. W, ORR, N W M, BOTTINO D, IKIN E. W, MOURANT A. E., and LEHMANN, H The blood groups and haemoglobin of the Bedouin of Socotra. Man 1 82–86 (1966)
16 LIVINGSTONE, F B., Malaria and human polymorphisms, in ROMAN, SANDLER and CAMPBELL Annual review of genetics, pp 33–64 (Annual Reviews, Palo Alto 1971)
17 MARGOLIS, E., GUREVITCH, J., and HERMONI, D Blood groups in Sephardic Jews. Am J phys. Anthrop 18 197–199 (1960)
18 McGREW, C. J., jr., Sickle cell trait in the white population. J Am. med. Ass. 224 1762–1763 (1973)
19 MOSES, W S MATHIOTH, Y., and LEVIN S. Sickle cell disease in Israel. Report of 5 cases in an Arab family Harefuah 51 281 (1956)
20 MOURANT, A. E. The distribution of the human blood groups, p 438 (Blackwell, Oxford 1954)
21 RACE, R. R and SANGER R., Blood groups in man pp 178–464 (Davis, Philadelphia 1968)
22 RACHMILEWITZ, E. A. LEVENE, C., HERSHKO C., HUTMAN T H J, FREUNDLICH, E., and SANDLER S. G Negro blood group markers and 'relatively benign' sickle cell disease in Israel. Abstract. Proc. 1st nat. Sickle Cell Disease Conf., Bethesda 1974 (in press)
23 RACHMILEWITZ E. A LEVY, S., and HUTMAN T H J High frequency of hemoglobin C in an Israel Bedouin tribe Israel J med Scis 10 219–224 (1974)
24 RAMOT B and SHEBA, C. H The genetics of Israel's populations, in GOLDSCHMIDT Genetics of migrant and isolated populations, pp 253–355 (Williams & Wilkins, Baltimore 1963).

CYRIL LEVENE, MD Director Reference Laboratory of Immunohematology and Blood Groups, Government Central Laboratories, Ministry of Health, 86 Yaffo Road, PO Box 6115 Jerusalem (Israel)

Acta haemat. 55 306-312 (1976)

Effect of Hyperoxia and Androgen on Red Cell 2,3-Diphosphoglycerate and Oxygen Affinity[1]

DOV GORSHEIN, FRANK H GARDNER, WARNER TYREE, FRANK OSKI and MARIA DELIVORIA-PAPADOPOULOS

Presbyterian University of Pennsylvania Medical Center, Philadelphia, Pa.

Key Words Androgens 2 3 Diphosphoglycerate Erythropoietin Hyperoxia Oxygen dissociation Oxyhemoglobin affinity

Abstract Increased plasma erythropoietin (ESF) activity, a rise in RBC 2,3-DPG, and a 'right shift' of the oxygen hemoglobin equilibrium curve following androgen administration to mice in ambient or hyperoxic conditions was demonstrated If androgens had a direct effect on the RBC metabolism increased 2,3-DPG would result in a facilitated release of oxygen to the tissue This would have been accompanied by a decrease rather than an increase in the level of ESF Hyperoxia has abolished detectable rising levels of plasma ESF and RBC 2 3 DPG following androgen administration These levels were close to those seen in the ambient nontreated mice

The erythropoietic capacity of androgens has long been recognized [13, 16, 35] It has been clearly demonstrated [1, 13] that androgens are capable of stimulating erythropoietin (ESF) production Recent studies reported a rise in RBC 2,3 diphosphoglycerate (2,3-DPG) and a 'right shift' of the oxygen hemoglobin equilibrium curve in hypoxic exposure [41] or following androgen administration in monkeys [18] and in patients with chronic renal failure maintained on bi-weekly dialyses [42] Two mechanisms may be proposed to account for this rise in RBC 2,3-DPG following androgen administration a direct effect of the androgen on the metabolism of the RBC, and/or relative tissue hypoxia. Hyperoxia has been reported to suppress RBC 2,3-DPG levels [25] The experi-

[1] This research was supported in part by grants from the John A Hartford Foundation, Inc, the Department of Health, Education and Welfare (AM 11188-06), and the United States Public Health Service No 713502-5-25263

ments reported here were designed to determine the effect of hyperoxia on the changes noted in plasma ESF levels, RBC 2,3-DPG, and the oxygen hemoglobin equilibrium curve following androgen administration.

Materials and Methods

Animals Swiss Webster white virgin female mice (West Jersey Biological Co., Wenonah NJ) randomized in groups of ten, were used and supplied with Purina Rodent Chow and water, *ad libitum* The mice received 1–2 mg of iron as iron-dextran solution (Imferon®, Lakeside Laboratories, Milwaukee, Wisc.) intraperitoneally to insure adequate iron stores.

Hyperoxic chamber Endogenous ESF, as measured by radioiron incorporation into peripheral RBC and reticulocyte counts [3] was suppressed by maintaining mice in a modified atmosphere chamber (Piersol Pine Manufacturing Co., Oaks, Pa) at 60% oxygen concentration and normal atmospheric pressure [27] Temperature was maintained as close as possible to 22 °C. The exposure to hyperoxia was interrupted daily for 10–15 min cage cleaning and injections.

Pooled blood from a group of 7 mice was obtained following 6 days exposure to hyperoxia and 48 h after the second 19-nortestosterone decanoate (19-ND) injection and RBC 2,3-DPG and the partial pressure at which 50% of the hemoglobin is oxygenated (P_{50}) were measured according to the method of DELIVORIA PAPADOPOULOS *et al* [7] Nortestosterone decanoate was kindly supplied by Dr HENRY STRADE (Organon West Orange, NJ) in a concentration of 25 mg/ml dissolved in propylene glycol Injections were given on the 4th and 5th hyperoxic day

Determination of plasma erythropoietic activity in the posthypoxic plethora. Erythropoietic activity in plasma of mice at ambient pressure was determined 1 h after the administration of a single injection of 2.5 mg 19 ND and compared to the activity 3 days later Plasma for ESF determination was also obtained from hyperoxic mice 1 2, 3, and 4 days after two daily administrations each of 2.5 mg 19 ND dissolved in 0 1 ml of propylene glycol Plasma ESF levels were measured in the post hypoxic polycythemic mouse system by injecting plasma samples of 0.5 or 1.0 ml each to mice on the 3rd and 5th hypoxic days, and 72 h radioiron incorporation was begun on the 6th posthypoxic day as previously described [19 23]

Results

The results of ^{59}Fe incorporation into RBC of posthypoxic polycythemic mice (table I) clearly indicate the lack of ESF activity of plasma obtained 1, 2, 3, and 4 days after 19 ND administration to mice maintained in hyperoxia. Radioiron incorporation into RBC of posthypoxic polycythemic mice following administration of plasma obtained from mice maintained in normal conditions and injected with 19 ND 1 h or 3

Table I Percent ^{59}Fe incorporation in posthypoxic polycythemic mice following administration of plasma obtained from mice maintained in hyperoxia and injected with 19-ND

Plasma obtained, days after 2nd 19-ND injection	Amount of plasma injected each time, ml	^{59}Fe incorporation, %[1]
1	0 5	2 5±0 7
	1 0	2.6±0 8
2	0 5	1.9±0 6
	1 0	3 0±1 0
	0 5	1.9±0 4
3	1 0	1 7±0 7
4	0 5	1 7±0 7
	1 0	2 2±0 5
Control[2]		2 0±1 2
0 25 U ESF[3]		4 1±0 3

1 Results expressed as mean±SEM of a group of at least eight animals
2 Injection of diluent only
3 Injected each time as for plasma administration

Table II RBC 2,3-DPG and P_{50} values in ambient and hyperoxic mice before and following 19-ND administration

		2,3-DPG, nM/ml RBC	P_{50} mm Hg, pH 7 4	DPG[1]	P_{50}[1]	DPG[2]	P_{50}[2]
Before	ambient	8,900±157	40 5±0 25	<0 05			
19-ND	hyperoxic	6,100±203	39 0±0 25		<0 1		
Following	ambient	10,250±215	42.5±0 25	<0 05		<0 05	
19-ND	hyperoxic	9,050±250	41 0±0 25		<0 1		<0 05

Each point represents mean of at least four measurements ±SEM, each measurement performed on pooled blood

1 Statistical significance of change in RBC, 2,3-DPG levels or P_{50} position following hyperoxic exposure

2 Statistical significance of change in RBC 2,3-DPG levels or P_{50} position following administration of 19 ND to mice in hyperoxic exposure

days before collection of plasma were previously reported [20] The results of the RBC 2,3 DPG and P_{50} (table II), clearly indicate a decrease in RBC 2,3 DPG as previously reported [25] along with a 'shift to the left' of the oxygen hemoglobin equilibrium curve following hyperoxic exposure A 'right shift' of the oxygen dissociation curve along with a rise in RBC 2,3-DPG occurs following the administration of 19 ND to ambient mice or mice maintained in hyperoxia

Discussion

The mechanism by which androgenic steroids exert their effects on hematopoiesis has been of continued interest in view of their clinical application A moderate increase of the red cell mass has been observed in rodents following injection of these steroids [28, 38, 39, 44], but after prolonged administration a refractory state occurs [17], perhaps related to the dampening effect of the increased red cell mass on the production of ESF The effect of androgens on ESF production is well documented both in animals and in man [1, 14, 17, 26, 34] Recently 19 ND has been reported to be a strong erythropoietic stimulant in mice [20] and in man [3] The mechanism by which ESF production is stimulated by androgens is not clear Hypoxic exposure can stimulate ESF production even in a nephrectomized state [2, 14, 35, 43] Tissue hypoxia [41] or hypoxic exposure [40] may be compensated for by a 'right shift' of the oxygen dissociation curve [41] along with an increase of RBC 2,3 DPG Hyperoxia exerts a suppressive effect on the ESF level [22, 31] and has also been reported to suppress the RBC 2 3 DPG level [25]

The relationship of androgen administration to the 'right shift' of the oxygen hemoglobin equilibrium curve and the rise in RBC 2,3 DPG has been previously noted [18, 37] An effect of the androgen on the RBC metabolism resulting in an increased level of 2,3 DPG has been reported [37] However, it is somewhat difficult to reconcile this concept with the current observations A primary increase of RBC 2 3 DPG results in facilitated release of oxygen to the tissue therefore, the level of ESF may not be expected to rise This is not the case in the present studies The androgen 19 ND has increased both the level of ESF and the RBC 2,3 DPG and caused an increase in the P_{50} ('right shift' of the oxygen hemoglobin dissociation curve) [18] When the mice were exposed to an increased oxygen tension the level of RBC 2,3 DPG decreased and the position of the

oxygen hemoglobin curve shifted to the left as expected [41] Androgen administration in hyperoxia resulted in an increase of the RBC 2 3 DPG and a shift of the oxygen hemoglobin equilibrium curve close to levels seen in the untreated ambient animal Thus it is not surprising that an associated increased level of ESF in animals exposed to hyperoxia and administered androgen was not observed Lack of detectable increase in plasma ESF following 19 ND administration in mice maintained in hyperoxia was not related to the presence of an inhibitor [9] as previously reported [12] Thus, it appears that the change in RBC 2 3 DPG following androgen administration is related to the level of tissue oxygenation rather than to the result of a primary effect on the RBC metabolism

References

1 ALEXANIAN R Erythropoietin and erythropoiesis in anemic man following androgens Blood *33* 564–572 (1969)
2 BACIU I OPRISIU C DORFTEIU M ROSENFELD E. RUSIU M et CHEREBETIU C. Le rein et la production de l érythropoïétine J Physiol Paris *55* 551–567 (1963)
3 BESA E C GORSHEIN D and GARDNER F H Blood volume changes in normal and various anemic states in man following androgens Archs intern Med *133* 418–425 (1974)
4 BYRON J Effect of steroids on the cycling of haemopoietic stem cells Nature Lond *228* 2104 (1970)
5 BYRON J Effect of steroids and dibutyryl cyclic AMP on the sensitivity of haematopoietic stem cells to ^{3}H thymidine *in vitro* Nature Lond *234* 39–40 (1971)
6 DEGOWIN R LAVENDER A FORLAND M CHARLESTON D., and GOTTSCHALK, A Erythropoiesis in an anephric patient treated with hemodialysis and testosterone Ann intern Med *72* 913–918 (1970)
7 DELIVORIA PAPADOPOULOS M RONCEVIC N and OSKI F Postnatal changes in oxygen transport of term immature and sick infants The role of red cell 2,3-diphosphoglycerate and adult hemoglobin. Pediat Res *5* 235–245 (1971)
8 DINGLE, J HEATH J WEBB M., and DANIEL, M The biological action of cobalt and other metals II The mechanism of the respiratory inhibition produced by cobalt in mammalian tissues Biochem biophys Acta *65* 34–43 (1962)
9 DONATI R and GALLAGHER N Antierythropoietic effect of plasma from hyperoxic rats Abstract Clin Res *17* 30 (1969)
10 FEIG S and SEGEL, G Androgens, 2,3 DPG and sickling New Engl J Med *287* 1097–1098 (1972)
11 FINKELSTEIN G GORDON A., and CHARIPPER H The effect of sex hormones on the anemia produced by hemorrhage in the rat. Endocrinology *35* 267–277 (1944)

12 Fishman, D., Leonard, R.; Gorshein, D., Besa, E.; Jepson, J., and Gardner, F.: Stem cell damage induced by hyperoxia. Biomedicine *19*: 291–295 (1973)

13 Fried, W. and Gurney, C.: The erythropoietic-stimulating effects of androgens. Ann. N.Y. Acad. Sci. *149*: 356–365 (1968)

14 Fried, W. and Kilbridge, T.: Effect of testosterone and of cobalt on erythropoietin production by anephric rats. J. Lab. clin. Med. *74*: 623–629 (1969)

15 Gallagher, N., McCarthy, J., and Lange, R.: Erythropoietin production in uremic rabbits. J. Lab. clin. Med. *57*: 281–289 (1961)

16 Gordon, A.: Endocrine influences upon the formed elements of blood and blood forming organs. Recent Prog. Horm. Res. *10*: 339–394 (1954)

17 Gordon, A.; Mirand, E.; Wenig, J.; Katz, R., and Zanjani, E.: Androgen actions on erythropoiesis. Ann. N.Y. Acad. Sci. *149*: 318–335 (1968)

18 Gorshein, D., Gardner, F., Oski, F., and Delivoria Papadopoulos, M.: Effect of androgens on the red cell 2,3-diphosphoglycerate hemoglobin oxygen affinity and red cell mass in mammals. Proc. Soc. exp. Biol. Med. (in press, 1974)

19 Gorshein, D. and Gardner, F.: Erythropoietic activity of steroid metabolites in mice. Proc. natn. Acad. Sci. USA *65*: 564–568 (1970)

20 Gorshein, D., Murphy, S., and Gardner, F.: Comparative study on the erythropoietic effects of androgens and their mode of action. J. appl. Physiol. *35*: 376–378 (1973)

21 Gorshein, D., Tyree, W., Hait, W.; Besa, E.; Gardner, F., and Delivoria Papadopoulos, M.: The effect of hyperoxia and androgen administration on erythrocyte 2,3-diphosphoglycerate level and oxygen affinity in mice. Abstract. Clin. Res. *21*: 556 (1973)

22 Gorshein, D., Hait, W., Besa, E., Jepson, J., and Gardner, F.: Rapid stem cell differentiation induced by 19-nortestosterone decanoate. Br. J. Haemat. *25*: 215–225 (1974)

23 Gorshein, D., Murphy, S., and Gardner, F.: Studies on the erythropoietic activity of steroid metabolites. Expl Hemat. *1*: 123–126 (1973)

24 Gorshein, D., Hait, W., Besa, E., Jepson, J., and Gardner, F.: Increased stem cell response to erythropoietin induced by androgens. Endocrinology *93*: 777–780 (1973)

25 Gaffney, H., Jefferson, D., and Mendoza, C.: Effect of hyperoxia on red blood cell (RBC) 2,3-diphosphoglycerate (2,3-DPG). Abstract. J. Lab. clin. Med. *78*: 841 (1972)

26 Gurney, C., Fried, W., and Fessler, A.: The erythropoietic effect of androgens; in Stohlman Hemopoietic cellular proliferation, pp. 133–140 (Grune & Stratton, New York 1970)

27 Hait, W., Gorshein, D., Jepson, J., Besa, E., and Gardner, F.: 19-Nortestosterone decanoate induced erythropoiesis in the actinomycin D treated mouse. Biochem. biophys. Res. Commun. *47*: 425–431 (1972)

28 Jepson, J. and Lowenstein, L.: Effect of prolactin on erythropoiesis in the mouse. Blood *24*: 726–738 (1964)

29 Jepson, J. and Lowenstein, L.: The effect of testosterone, adrenal steroids, and prolactin on erythropoiesis. Acta haemat. *38*: 292–299 (1967)

30 Jepson, J. and Lowenstein, L.: Inhibition of the stem cell action of erythropoie-

oxygen hemoglobin curve shifted to the left as expected [41] Androgen administration in hyperoxia resulted in an increase of the RBC 2 3 DPG and a shift of the oxygen hemoglobin equilibrium curve close to levels seen in the untreated ambient animal Thus, it is not surprising that an associated increased level of ESF in animals exposed to hyperoxia and administered androgen was not observed Lack of detectable increase in plasma ESF following 19 ND administration in mice maintained in hyperoxia was not related to the presence of an inhibitor [9] as previously reported [12] Thus, it appears that the change in RBC 2,3 DPG following androgen administration is related to the level of tissue oxygenation rather than to the result of a primary effect on the RBC metabolism

References

1 Alexanian R Erythropoietin and erythropoiesis in anemic man following androgens Blood *33* 564–572 (1969)

2 Baciu I Oprisiu C. Dorfteiu M Rosenfeld E. Rusiu M et Cherebetiu C. Le rein et la production de l érythropoïétine J Physiol Paris *55* 551–567 (1963)

3 Besa E. C , Gorshein D and Gardner F H Blood volume changes in normal and various anemic states in man following androgens Archs intern Med *133* 418–425 (1974)

4 Byron J Effect of steroids on the cycling of haemopoietic stem cells Nature Lond *228* 2104 (1970)

5 Byron J Effect of steroids and dibutyryl cyclic AMP on the sensitivity of haematopoietic stem cells to ^{3}H thymidine *in vitro* Nature Lond *234* 39–40 (1971)

6 DeGowin R Lavender A Forland M Charleston D and Gottschalk, A Erythropoiesis in an anephric patient treated with hemodialysis and testosterone Ann intern Med *72* 913–918 (1970)

7 Delivoria Papadopoulos M Roncevic N., and Oski F Postnatal changes in oxygen transport of term immature and sick infants The role of red cell 2 3-diphosphoglycerate and adult hemoglobin Pediat. Res *5* 235–245 (1971)

8 Dingle, J Heath J Webb M., and Daniel, M The biological action of cobalt and other metals II The mechanism of the respiratory inhibition produced by cobalt in mammalian tissues Biochem biophys Acta *65* 34–43 (1962)

9 Donati R and Gallagher N Antierythropoietic effect of plasma from hyperoxic rats Abstract Clin Res *17* 30 (1969)

10 Feig S and Segel, G Androgens, 2 3 DPG and sickling New Engl J Med *287* 1097–1098 (1972)

11 Finkelstein G Gordon A., and Charipper H The effect of sex hormones on the anemia produced by hemorrhage in the rat. Endocrinology *35* 267–277 (1944)

12 Fishman D, Leonard, R., Gorshein D, Besa, E., Jepson J., and Gardner, F.. Stem cell damage induced by hyperoxia. Biomedicine *19* 291–295 (1973)
13 Fried, W and Gurney, C. The erythropoietic-stimulating effects of androgens. Ann N Y Acad Sci. *149* 356–365 (1968)
14 Fried W and Kilbridge, T Effect of testosterone and of cobalt on erythropoietin production by anephric rats J Lab clin. Med. *74* 623–629 (1969)
15 Gallagher, N, McCarthy, J., and Lange, R. Erythropoietin production in uremic rabbits. J Lab clin Med. *57* 281–289 (1961)
16 Gordon, A. Endocrine influences upon the formed elements of blood and blood forming organs Recent Prog Horm Res. *10* 339–394 (1954)
17 Gordon A., Mirand E., Wenig J, Katz, R., and Zanjani, E. Androgen actions on erythropoiesis. Ann. N Y Acad Sci *149* 318–335 (1968)
18 Gorshein D, Gardner F, Oski F., and Delivoria Papadopoulos, M Effect of androgens on the red cell 2,3-d phosphoglycerate hemoglobin oxygen affinity and red cell mass in mammals. Proc. Soc exp Biol Med (in press, 1974)
19 Gorshein, D and Gardner, F Erythropoietic activity of steroid metabolites in mice Proc natn Acad. Sci. USA *65* 564–568 (1970)
20 Gorshein D Murphy, S., and Gardner, F Comparative study on the erythropoietic effects of androgens and their mode of action. J appl Physiol *35* 376–378 (1973)
21 Gorshein D Tyree, W, Hait, W, Besa E, Gardner F., and Delivoria Papadopoulos M The effect of hyperoxia and androgen administration on erythrocyte 2,3-diphosphoglycerate level and oxygen affinity in mice Abstract. Clin Res *21* 556 (1973)
22 Gorshein D Hait W Besa, E., Jepson, J., and Gardner, F Rapid stem cell differentiation induced by 19 nortestosterone decanoate Br J Haemat. *26* 215–225 (1974)
23 Gorshein D, Murphy S., and Gardner, F.. Studies on the erythropoietic activity of steroid metabolites. Expl Hemat. *1* 123–126 (1973)
24 Gorshein D, Hait, W, Besa, E., Jepson J., and Gardner, F Increased stem cell response to erythropoietin induced by androgens Endocrinology *93* 777–780 (1973)
25 Greene, H Jefferson D., and Mengal, C.. Effect of hyperoxia on red blood cell (RBC) 2,3-d phosphoglycerate (2,3-DPG) Abstract. J Lab clin. Med. *78* 841 (1972)
26 Gurney, C., Fried W., and Fessler, A The erythropoietic effect of androgens in Stohlman Hemopoietic cellular proliferation, pp 133–140 (Grune & Stratton New York 1970)
27 Hait W Gorshein, D, Jepson J Besa, E., and Gardner, F 19 Nortestosterone decanoate induced erythropoiesis in the actinomycin D-treated mouse Biochem biophys. Res. Commun. *47* 426–431 (1972)
28 Jepson J and Lowenstein L.. Effect of prolactin on erythropoiesis in the mouse Blood *24* 726–738 (1964)
29 Jepson J and Lowenstein L The effect of testosterone, adrenal steroids, and prolactin on erythropoiesis. Acta haemat. *4*? 292–299 (1967).
30 Jepson J and Lowenstein L. Inhibition of the stem cell action of erythropoie-

tin by estradiol valerate and the protective effect of 17α hydroxyprogesterone caproate and testosterone propionate Endocrinology *80* 430–434 (1967)
31 LINMAN, J and PIERRE, R Studies on the erythropoietic effect of hyperbaric hyperoxia Ann N Y Acad Sci *149* 25–33 (1968)
32 *LUNDH, B and GARDNER, F* · *The haematological response to androgens in sickle* cell anaemia Scand J Haematol 7 389–397 (1970)
33 MILLER, W , DELIVORIA PAPADOPOULOS M , MILLER, L, and OSKI, F Increased release of oxygen from hemoglobin in hyperthyroidism Control by red cell 2 3 diphosphoglycerate J Am med Ass *211* 1824–1826 (1970)
34 MIRAND, E ; GORDON, A , and WENIG, J Mechanism of testosterone action in erythropoiesis Nature, Lond *206* 270–272 (1965)
35 *MIRAND, E., MURPHY, G , STEEVES, R , GROENEWALD, J , ZYL, J VAN, and RE-TIEF, F* · Erythropoietin alterations in renoprival and renal allografted patients Proc Soc exp Biol Med *129* 824–827 (1968)
36 MIRAND, E., MURPHY, G , STEEVES, R , GROENEWALD, J., and DEKLERK, J Erythropoietin activity in anephric, allotransplanted, unilaterally nephrectomized, and intact man J Lab clin Med *73* 121–128 (1969)
37 MOLINARI, P , CHUNG, S , and SNYDER, L Variations of erythrocyte glycolysis following androgens J Lab clin *Med 81 443–446 (1974)*
38 NAETS J and WITTEK M Mechanism of action of androgens on erythropoiesis Am J Physiol *210* 315–320 (1966)
39 NAETS, J and WITTEK, M The mechanism of action of androgens on erythropoiesis Ann N Y Acad Sci *149* 366–376 (1968)
40 OSKI, F , GOTTLIEB, A , DELIVORIA PAPADOPOULOS, M , and MILLER, W Red-cell 2,3 diphosphoglycerate levels in subjects with chronic hypoxemia New Engl J Med *280 1165–1166 (1969)*
41 OSKI, F and DELIVORIA PAPADOPOULOS, M The red cell, 2,3 diphosphoglycerate, and tissue oxygen release J Pediat. *77* 941–956 (1970)
42 PARKER, J ; BEIRNE, G , DESAI, J , RAICH, P., and SHAHIDI, N Androgen induced increase in red-cell 2,3-diphosphoglycerate New Engl J Med 287 381–383 (1972)
43 PILIERO, S ; MEDICI, P , and GORDON, A Production of the circulation erythropoietic factor in hypoxic endocrine deficient rats *Proc 7th Congr Int Soc* Hematol., part I, vol 2, pp 267–274 (1958)
44 RAO, L. and SHAHIDI, N The effect of 11 ketopregnenolone (*11 KP*) on red cell volume (RCV) Abstract Pediat Res *6* 372 (1972)
45 THORLING, E. and ERSLEV, A · The effect of some erythropoietic agents on the 'tissue' tension of oxygen Br J Haemat *23* 483–490 (1972)

DOV GORSHEIN, MD, 1900 South Broad Street, Saint Agnes Hospital, *Philadelphia PA 19145* (USA)

Acta haemat. 55 313-319 (1976)

A Second Case of Haemoglobin Belfast (β 15 [A 12] Trp→Arg) Observed in a French Patient

G. GACON, H. WAJCMAN, D. LABIE, B. VARET and B. CHRISTOFOROV

Institut de Pathologie Moléculaire Inserm U 15, Laboratoire Associé au CNRS
Service d'Hématologie, and Service de Médecine, Hôpital Cochin, Paris

Key Words: Haemoglobin Belfast · Haemoglobinopathies · Oxygen dissociation · Unstable haemoglobins

Abstract. Haemoglobin Belfast was initially observed in an Irish patient. The second case, found in a French patient suffering from a metastatic carcinoma, is here described. Both observations are compared from clinical, haematological and biochemical points of view. Functional and spectrophotometrical studies confirmed the slight abnormalities already described.

Among the unstable haemoglobins, some give rise to severe haematological disorders, others are only discovered during a systematic survey or routine examination. Haemoglobin Belfast (β 15 [A 12] Trp → Arg) apparently belongs to this last group. It was described in an Irish long-stay psychiatric patient with polycystic kidneys [9]. We report here the second observation of this abnormal haemoglobin, incidentally found in a French patient suffering from a metastatic cancer.

In all the known haemoglobins the residue at position A 12 is an hydrophobic one, usually tryptophan [6], acting as a spacer between helices A and E [12]. As a consequence of the substitution of an arginine for this tryptophan, a destabilization of the globin structure and a slight increase of the oxygen affinity were described.

Material and Methods

Haematological studies were done following routine procedures.

Examination of the haemolysate by electrophoresis was carried out using acetate-cellulose plates (Titan III, Helena Laboratories) in Tris EDTA-borate buffer, pH

Isoelectrofocusing on polyacrylamide gels was done according to the technique of DRYSDALE *et al* [7]

The isopropanol stability test was performed as described by CARREL and KAY [3]

The abnormal component was isolated and quantitated by DEAE Sephadex chromatography using a linear gradient of Tris HCl 0 05 M buffer from pH 8 0 to pH 7.3 The globin chains were separated by a Clegg's column [4] The tryptic digest of the amino-ethylated β chain was analyzed by finger printing on silica gel thin layer Specific stainings were used for the identification of the spots [8]

The oxygen delivery function was studied on intact red blood cells (RBC) by the discontinuous spectrophotometric technique of BENESCH *et al* [2] in isotonic phosphate buffer, pH 7 15 [1] The intra-erythrocytic 2,3-diphosphoglycerate (2,3 DPG) was measured enzymatically following the formation of NADH at 340 nm (Sigma Technical Bulletin 35 UV) The lysate and the pure components were made free of organic phosphates by ion exchange chromatography [11], and the oxygen affinity was measured in 2,2 bis (hydroxymethyl) 2-2-2 -nitriloethanol (bis tris), 0 05 M buffer at 25 °C The pH dependence of log P_{50} at 25 °C was measured in bis tris 0 05 M NaCl 0 1 M buffer below pH 7.5 and in Tris HCl 0 05 M, NaCl 0 1 M buffer above this pH value

Spectrophotometric studies were performed in 0 15 M phosphate buffer, pH 6.5, on a Beckman Acta III spectrophotometer

Case Report

M F., a 53 year-old French man, was hospitalized for thoracic pain He had no previous pathological history and did not remember of any haemoglobin study He was the second child of unrelated French parents without known Celtic ancestry, both deceased Physical examination showed no abnormality apart from a painful tumefaction at the D 7 level and 9th right rib Routine blood count performed on a Coulter Counter model S showed an anaemia (see below), and a haemoglobin electrophoresis was routinely performed

A surgical biopsy of the 9th right rib showed a tumoral bone lesion. At histological examination, a diagnosis of metastatic carcinoma of poorly differentiated type was made. No primitive carcinoma was discovered after numerous radiological investigations including normal intravenous pyelogramm and renal arteriography

The patient was not yet treated when the haematological and biochemical studies were done

Haematological studies The blood film showed no abnormalities apart from hypochromia Haemoglobin 8.5 g/100 ml, red cells $3\,18 \times 10^6/\mu l$, packed cell volume 27 4%, mean corpuscular haemoglobin concentration 31 4%, mean corpuscular volume 85 μm^3, reticulocyte count 45,000/μl without inclusion bodies on the smear

Serum iron level was 15 μg/100 ml with a total iron binding capacity of 135 μg/100 ml These results and the high erythrocyte sedimentation rate favour the diagnosis of inflammatory anaemia in relation with widespread carcinoma Serum bilirubin and haptoglobin were normal

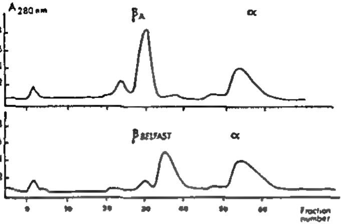


Fig. 1 Comparison between elution patterns of chain separation in Hb Belfast and Hb A

Numerous Heinz bodies were shown in the red cells after 24 h incubation at 37 °C and staining with methylviolet [5]

Haemoglobin Study

An abnormal haemoglobin migrating like Hb S was observed during routine electrophoresis. The solubility test was normal but an instability was observed within 30 min.

The isoelectric point of the abnormal component, as determined by electrofocusing on polyacrylamide gel, was 7.23, the same as for Hb S and Hb D Punjab. The percentage of the different haemoglobins was: Hb A_2 2.7%, Hb A_1 68%, and Hb Belfast 29%.

The elution pattern of the Clegg's column showed two abnormalities: a β-chain eluted later than normal and a modified absorbance ratio at 280 nm between β- and α-chains. It was equal to 0.9 instead of 1.5 (fig. 1).

The peptide map was the same as described by KENNEDY *et al.* [9]. By ninhydrin staining there was no normal βT_2 and a faint βT_3. Three additional spots were seen (fig. 2). The specific stainings showed the disappearance of β 15 tryptophan and a positive staining for arginine of two new peptides corresponding, as described, to sequence β 9–15 and β 16–30. The third newly appeared spot was the dipeptide β 16–17.

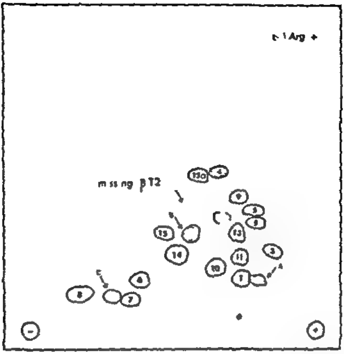


Fig. 2. The missing β T2 is in dotted lines. Three additional peptides are shown by arrows, corresponding to new tryptic cleavages. A = Residues 16 through 30: elongated β T3, without cleavage between residues 17 and 18. B = Residues 9 through 15: shortened β T2. C = Residues 16 and 17: cleavage at both Arg 15 and Lys 17.

The oxygen dissociation curve of the patient's intact RBC was shifted to the right, the P_{50} being 35 mm Hg (normal = 29.5 ± 1.0), but the intraerythrocytic 2,3-DPG was very high (24 μmol/g Hb instead of 13 ± 1). When compared to normal RBC containing the same amount of 2,3-DPG, the extent of the shift is in the normal range [10]. On the stripped total lysate, the P_{50} was 2.2 mm Hg. This value is not significantly different from the normal.

On the pure abnormal component, an increase of the oxygen affinity of about 10% was found. In bis-tris 0.05 M, pH 7.15 buffer, the P_{50} values were 1.88 mm Hg for Hb Belfast and 2.04 for Hb A_1. The same being observed at various pH values, the pH dependence curves of log P_{50} were parallel, indicating an identical Bohr effect together with a higher oxygen affinity of the abnormal haemoglobin (fig. 3). The co-operativity was always found normal. The addition of known amounts of 2,3-DPG into the tonometer shifted the oxygen dissociation curve in an identical way for both haemoglobins.

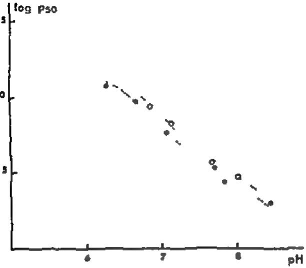


Fig 3 pH dependence of the oxygen affinity ○ = Phosphate-free pure Hb A. ● = phosphate-free pure Hb Belfast.

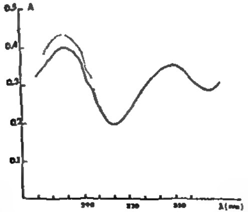


Fig 4 Ultra violet absorption spectrum of oxyhaemoglobin Belfast (——) and oxyhaemoglobin A (-----). A difference is observed only in the ultra-violet part of the spectrum.

As expected from the disappearance of a tryptophan residue, spectrophotometrical abnormalities were observed. When comparing the optical ultra-violet spectrum of the abnormal haemoglobin to normal, at the same concentration, a clear decrease of the absorbance was observed (fig. 4).

Discussion

This case is the second observation of Hb Belfast. It was found in a patient of French origin (Burgundy) and no relation could be found with the previously described one. In both cases an identical mutation may have occurred independently. In the first description, the abnormal haemoglobin was associated with hereditary polycystic kidneys, and the haematological disorders were moderate. In our patient, the kidneys were found normal and the severe anaemia observed had all the characteristics of a chronic inflammatory process, very likely due to the progress of the cancer. No obvious symptoms of haemolysis could be correlated to the presence of an unstable haemoglobin. The proportion of the abnormal component is close to what was described in the observation of KENNEDY *et al.* [9]. These authors showed that the ratio of synthesis was identical for Hb Belfast and Hb A in the reticulocytes, these results excluding the hypothesis that the iron deficiency found in both cases may explain a defective synthesis of the abnormal haemoglobin.

From both observations, haemoglobin Belfast seems to be moderately unstable. Nevertheless, some ambiguity remains due to the association of a severe disease which could explain non specific haematological disorders.

The structural abnormality was easily identified by analytical fingerprint on silica gel thin-layer due to the identity with an earlier description. Our findings concerning the oxygen equilibrium confirm the 'slight but significant' increase of the oxygen affinity previously described on the total lysate. On the pure abnormal component, the oxygen affinity is clearly increased by about 10%. Both this small difference in the oxygen delivery properties and the low percentage of Hb Belfast present in the cells explain why the alterations of the oxygen affinity are minimal in the total lysate and in the intact RBC.

Acknowledgements. This work was supported by grants from the 'Institut National de la Santé et de la Recherche Médicale' and the 'Centre National de la Recherche Scientifique'.

References

1 BELLINGHAM, A. J. and HUEHNS, E. R. Compensation in haemolytic anaemias caused by abnormal haemoglobins. Nature, Lond *218* 924–926 (1968)

2 *BENESCH, R., MAC DUFF, G., and BENESCH, R. E.* Determination of oxygen equilibria with a versatile new tonometer. Analyt. Biochem *11* 81–87 (1965)

3 CARRELL, R. W. and KAY, R. A simple method for the detection of unstable hemoglobins. *Br. J. Haemat. 23 615–619 (1972)*

4 CLEGG, J. B., NAUGHTON, M. A., and WEATHERALL, D. J. Abnormal human hemoglobins. Separation and characterization of the α and β chains by chromatography and the determination of two variants Hb Chesapeake and Hb J Bangkok. J. molec. Biol *19* 91–108 (1966)

5 DACIE, J. V. and LEWIS, S. M. Practical haematology (Churchill, London 1968)

6 DAYHOFF, M. O. Atlas of protein and structure, vol. 5 (National Biomedical Res. Found., Silver Spring 1972)

7 DRYSDALE, J. W., RIGHETTI, P. G., and BUNN, H. F. The separation of human and animal hemoglobins by isoelectric focusing in polyacrylamide gel. Biochim. biophys. Acta *229* 42–50 (1971)

8 EASLEY, C. W., ZEGERS, B. J. M., and DE VIJLDER, M. Application of specialized techniques for specific staining of peptide maps on various media. Biochim. biophys. Acta *175* 211–213 (1969)

9 KENNEDY, C. C., BLUNDELL, G., LORKIN, P. A., LANG, A., and LEHMANN, H. Haemoglobin Belfast 15 (A 12) tryptophan → arginine: a new unstable haemoglobin variant. Br. med. J. *iv* 324–326 (1974)

10 LABIE, D., BERNADOU, A., WAJCMAN, H. et BILSKI PASQUIER, G. Observation familiale d'une hémoglobine Genova β28 (B10) Leu→Pro. Etude clinique, hématologique, génétique et biochimique d'une famille française. Nouv. Revue fr. Hémat *12* 502–506 (1972)

11 NOZAKI, Y. and TANFORD, C. Examination of titration behavior. Meth. Enzym., vol. 11, pp. 715–734 (Academic Press, New York 1967)

12 PERUTZ, M. F., MUIRHEAD, H., COX, J. M., and GOAMAN, L. C. G. Three-dimensional Fourier synthesis of Horse oxyhaemoglobin at 2.8 Å resolution: the atomic model. Nature, Lond. *219* 131–139 (1968)

Dr. DOMINIQUE LABIE, Institut de Pathologie Moléculaire, 24, rue du Faubourg Saint Jacques, *F-75014 Paris* (France)

L. CAPRINO and E. C. ROSSI (ed.) **Platelet Aggregation and Drugs.**
1974 £ 5.80/US $ 15.25

The proceedings of the 3rd Serono Symposium held in Rome contain 20 contributions distributed in 3 different chapters (1) Physiological and pathophysiological aspects of platelet aggregation, (2) Biochemical and pharmacological aspects of the platelet aggregation, and (3) Platelet aggregation control clinical potential. Even though during the last years many symposia and conferences have been dedicated to platelets and mainly to their aggregation and its inhibition, these proceedings contain some original contributions or at least some original aspects of the recent developments. Besides the more theoretical aspects of the platelet functions reviewed in the first and second chapters, the last chapter is devoted to practical problems which are of a more general interest to people in charge of patients and of their control. This part of the book is certainly interesting but it solves less problems than it creates. However, reading of *Platelet Aggregation and Drugs* can be recommended. The papers are clearly written and easy to follow. F. DUCKERT, *Basel*

M. W. STEWARD **Immunchemie.** Aus dem Englischen übersetzt von Dr. KLAUS ESSER. Stuttgart 1975. VIII + 88 pp., 27 fig., 18 tab., DM 9.80. ISBN 3-437-20144-1

Auf kaum 80 Seiten Kleinformat ist es dem Autor gelungen, uns einen ausgezeichneten, klaren Überblick über die Immunchemie zu geben. Neben der Struktur der Immunglobuline diskutiert er die physikochemischen Eigenschaften von Antigenen und die Grundlagen der Antigen-Antikorper-Reaktionen und der Komplementfixierung. Ausser gesicherten Kenntnissen werden viele offene Fragen und Ausblicke erwähnt. Eine ausgewählte Bibliographie, die bis 1974 reicht, hilft dem Leser den Zugang zur Originalliteratur zu finden. Die «Immunchemie» kann allen immunologisch interessierten Biologen, Studenten und Ärzten wärmstens empfohlen werden. T. L. VISCHER, *Genf*

Acta haemat 55 321–331 (1976)

Hemopoietic Recovery in Bone Marrow of Lethally Irradiated Rats Following Parabiosis

I Granulopoiesis

With 1 color plate

SATIMARU SENO, CHAO HUI FANG, SHIGERU HIMEI, CHAO LIANG HSUEH and YUTAKA NAKASHIMA

Department of Pathology, Okayama University Medical School, Okayama

Key Words Bone marrow recovery after irradiation · Granulopoiesis · Hemopoiesis · Irradiation · Parabiosis

Abstract Aplasia was induced in rats by total body irradiation. Three days later, the animal was conjugated by aortic anastomoses with a healthy untreated litter mate 6 h after parabiosis, the bone marrow of irradiated animals contained some granulocytes showing RNA synthesis. At 18 h, many myelocytes and promyelocytes were present but no myeloblast was encountered These myeloid precursor cells showed active DNA synthesis but no mitoses, and no erythroblasts were observed at this time period At 24 h, mitoses of myeloblasts were found At 42–60 h, erythropoiesis was evident Chromosome analysis and investigations of cells of irradiated parabionts conjugated with partners having labeled cells, revealed that these newly formed myeloid and erythroid cells originated from the untreated parabiont. The mechanism of triggering myelopoiesis in the aplastic bone marrow by parabiosis is discussed

It is generally held that the various kinds of blood cells originate from a stem cell with a multidifferentiating potential [1] TILL and MCCULLOCH [2] reported the presence of bone marrow stem cells forming colonies in the spleen Other investigators reported that stem cells were found in circulating blood [3, 4] BECKER *et al* [5] and BARNES *et al* [6] reported that each cell colony originated from a single stem cell Independent *in vitro* studies by PLUZNIK and SACKS [7] and BRADLEY and METCALF [8] demonstrated colony formation of bone marrow and spleen cells in culture However, the morphologic identity of the stem cell is not clearly established

In the present investigation, stem cell activity was examined in rats after irradiation and parabiosis by light microscopy, by chromosome analysis, and by radioautography

Materials and Methods

Animals Inbred adult Wistar rats weighing from 250 to 300 g were used (n = 262) They were divided into 5 groups 36 pairs of male male littermates for irradiation and parabiosis, 20 pairs of male female littermates for irradiation and parabiosis, 28 male irradiated (nonparabiosis) controls, 10 male nonirradiated (nonparabiosis) controls and 112 male aorta donors

Irradiation A single dose of total body irradiation at 1,000 rad, 200 kVP, 65 cm, was administered to one parabiont member (always male) prior to parabiosis Animals in the treated control group were exposed to the identical dose of irradiation

Parabiosis Three days after irradiation the irradiated member was joined in parabiosis with an untreated, healthy littermate Parabiosis was performed by the method of aortic anastomoses using donor aortas as extensions [9, 10] This procedure permitted the immediate and complete cross-circulation of blood between the

day that the prospective partner was subjected to irradiation) ^{3}HTdr (specific activity, 5 Ci/mM) was injected intravenously, 0.3 mCi/100 g body weight once

Chromosome analysis and mitotic index At 3 h prior to sacrifice the male female parabionts were administered colchicine subcutaneously at 0 1 mg/100 g body weight Sex chromosome analysis by karyotype was performed with sedimented bone marrow cells using the method of OMURA [11] with 32–87 chromosome sets being analyzed in one sample The mitotic indices were obtained from irradiated animals by counting 2 000 sedimented nucleated cells

Animal sacrifice The animals were sacrificed by bloodletting The male male parabionts were sacrificed at 3, 6, 12, 18, 24 30, 36 42 and 48 h after parabiosis The male female parabionts were sacrificed at 18, 24, 36 48, 60, and 120 h after parabiosis 3–4 pairs of parabionts were sacrificed at each of these time periods Animals administered ^{3}HTdr were sacrificed 18 h after parabiosis The irradiated control animals were sacrificed on day 3 4 5, and 6 following irradiation Animals found dead prior to sacrifice were discarded

Blood cell and tissue preparation At sacrifice about 10 ml of blood from the abdominal aorta was drawn heparinized and centrifuged at 1,300 rpm for 5 min, and the sedimented cells were smeared on slides and stained with May-Grünwald Giemsa From ^{3}HTdr administered animals, peripheral smear specimens were mounted with sensitive emulsion exposed for 1 month, developed and stained with May Grünwald Giemsa

The bone marrow tissues were examined on the femurs of all the animals except for the donors of aorta A small amount of tissue was fixed in 10% formol for par

affin sectioning and stained by hematoxylin-eosin The remainder of the collected tissue was mixed with an equal volume of the rat serum and crushed gently in a glass homogenizer ▪ free the hemopoietic cells from the tissue The cramped connective tissue was removed and the cell suspension was centrifuged at 1 000 rpm for 5 min the sedimented cells were smeared on slides and stained with May Grünwald-Giemsa. Some smears were stained with benzidine peroxidase reaction.

Nucleic acid syntheses in vitro The sedimented cells from each irradiated member of the male-male unions sacrificed at 6 12, and 18 h after parabiosis were divided into two parts one part was incubated with ^{3}H uridine (^{3}HUr) ▪ 10 μCi/ml and 1 mM of cold thymidine for 40 min, and the other part was incubated with $^{3}HTdr$ at 1 μCi ml for 40 min The incubation medium was 3 ml of Hanks solution with an equal volume of rat serum. After incubation, the cells were sedimented and washed in 10 ml of Hanks solution, smeared on slides, mounted with sensitive emulsion (Sakura NRM2) exposed for 3 weeks, and developed and stained with May Grünwald Giemsa

Morphologic studies were conducted on samples from all animals. Cell classification studies were performed on smears from all groups, except for the male female parabionts

Results

In irradiated controls the bone marrow was aplastic 3 days after irradiation Examination of bone marrow smears indicated that the nucleated cells were plasma cells and fibroblast like cells, or reticulum cells In some exceptional samples atypical myeloid and lymphoid cells were occasionally encountered Mitosis was found in about 0 3‰ of cells and these cells were probably interstitial cells and plasma cells as indicated by their morphologic feature Most animals died by 5 days after irradiation Those surviving for 6 or 7 days following irradiation showed neither myelocytes, mature granulocytes nor lymphoid cells indicating that the myeloid precursor cells found occasionally at 3 days after irradiation were destined to die and were without replicating potency

Recovery of hemopoiesis by parabiosis Irradiated animals conjugated by parabiosis showed almost complete hemopoietic recovery at 5 days following parabiosis Bone marrow tissue sections ▪ this time period showed active myelopoiesis and erythropoiesis One day after parabiosis myeloid precursor cells with mitoses were present but no erythroblast was found Two days after parabiosis, distinct erythropoiesis with active myelopoiesis was observed.

The mitotic index of bone marrow cells of irradiated parabionts was low ▪ h after parabiosis compared to the irradiated control group However, from day ▪ following parabiosis the mitotic index increased and at

Table I. Mitotic indices and number of female mitotic cells from the bone marrow of irradiated male rats joined to untreated females by parabiosis

Hours after parabiosis	Number of pairs	Mitotic index[1], % (mean±SD)	Number of mitotic cells		
			female cell number	cells counted	female cells/total cells, %
18	3	0.28±0.12	2	49	4.1
			2	55	3.6
			1	68	1.5
24	4	0.64±0.15	20	57	35.1
			13	50	26.0
			20	72	27.8
			18	59	30.5
36	3	0.95±0.10	30	59	50.8
			44	88	50.0
			22	43	51.2
48	3	1.20±0.15	30	59	50.8
			32	65	49.2
			34	71	47.9
60	4	2.16±2.70	41	52	78.8
			55	76	72.4
			16	32	50.0
			40	72	55.6
120	3	9.46±0.44	81	84	96.4
			57	60	95.0
			80	87	92.0
Irradiated control	3	0.32±0.06	–	–	–
Untreated partners	3	5.50±0.70	–	–	–

[1] The mitotic index was determined in each animal by counting 2,000 cells.

day 5 after parabiosis the value exceeded the level of the healthy untreated partner (table I).

Chromosome analysis on bone marrow cells of irradiated males conjugated with females revealed that a few female cells began mitosis in the male aplastic bone marrow 18 h after parabiosis, and these cells entered into active mitoses 24 h after parabiosis. Thereafter, the percentage of fe-

Table II Classification of cells collected from irradiated bone marrow of male-male parabionts (mean values from 3 or 4 pairs)

Cell groups	Healthy controls	Irradiated controls	Hours after parabiosis								
			3	6	12	[illegible]	24	30	36	42	48
Myeloid cells											
Mature cells	26.2	6.6	19.8	28.0	27.5	32.9	26.7	12.5	13.5	11.2	21.7
Myelocytes	4.0	0.1	0	0	1.7	3.8	1.8	0.5	2.0	3.2	3.0
Promyelocytes	1.8	0	0	0	0	4.2	3.5	3.0	1.5	2.5	2.0
Myeloblasts	0.4	0	0	0	0	0	0.2	0.5	1.0	1.2	0.5
Lymphoid cells	26.5	0.6	29.0	24.5	36.3	31.2	31.5	50.0	45.0	15.2	6.0
Monocytes	2.0	0.9	0.2	2.0	1.5	1.3	1.0	0.3	2.0	1.5	0.6
Plasma cells	0.8	72.9	29.4	21.5	28.2	22.9	32.2	24.0	24.0	38.9	8.5
Reticulum cells	5.0	15.9	21.5	22.5	4.3	3.5	2.5	9.0	10.5	17.5	1.5
Megakaryocytes	0.2	3.0	0.1	1.5	0.5	0.2	0.6	0.2	0.5	0.1	0.2
Erythroblasts	33.1	0	0	0	0	0	0	0	0	8.7	56.0

male mitotic cells increased to 50% after 48 h and to 95% after 120 h, indicating that hemopoietic recovery was made by cells originated from the healthy female partner (table I)

Morphologic observations on bone marrow cells in irradiated males conjugated with healthy males revealed that 3 h after parabiosis dramatic accumulations of granulocytes and lymphoid cells were found in bone marrow sections and smears These cell accumulations were not encountered before parabiosis Granulocytes constituted about 20% with lymphoid cells and plasma cells each constituted about 30% of nucleated cells, monocytes constituted less than 1% No erythroblasts were encountered Almost all granulocytes were mature and some metamyelocytes were present but no myelocytes, promyelocytes or myeloblasts were found (table II) Small and large lymphoid cells were found in about equal numbers

At 6 h after parabiosis, the frequency of the bone marrow cells was about the same as that seen 3 h after parabiosis. However, some large granulocytes appeared, 12–15 μm in diameter with a segmented or ring-form nucleus and these cells showed a slightly basophilic cytoplasm (fig 1a)

At 12 h after parabiosis the cellularity of the bone marrow increased.

The distinctive feature of this period was the appearance of some myelocytes (table II) These cells had a thick ring-form or penetrated nucleus, fine chromatin nets, moderately basophilic cytoplasm, and most cells contained many azurophilic granules (fig 1b) The cytoplasm indicated a strong positive peroxidase reaction (fig 1g) The percentage of monocytes remained small

At 18 h after parabiosis many younger myeloid precursor cells appeared (table II) Most of them were myelocytes and promyelocytes, though a few myeloblasts were found by careful observation The characteristic of this stage was the appearance of promyelocytes having a solid nucleus They were large in size, had a basophilic cytoplasm with many azurophilic granules (fig 1c) and gave a strong peroxidase reaction by *which they were distinguished from monocytes*

At 24 h after parabiosis the cellularity of the bone marrow continued to increase and some young myeloblasts appeared These cells showed a deeply basophilic cytoplasm and some azurophilic granules (fig 1d) Mitoses of myeloid cells were occasionally observed Granulocytic cells constituted about 30% of the total cell population, and the myeloid precursor cells occupied about 17% of the total granulocytic cells (table II)

Lymphoid cells occupied about 30% of the total nucleated cells during the period from 3 to 24 h *after parabiosis During this interval plasma* cells constituted about 30% of cells compared to the irradiated control value of 73%, and reticulum cells decreased from 21 5 to 2 5% Monocytes showed little change during this period (table II) The morphologic investigation failed to reveal cells suggestive of transition from lymphoid or monocytoid cells to myelocytes, promyelocytes, or myeloblasts

In the period 30 and 36 h after parabiosis, distinct myelopoiesis was present in the bone marrow tissues In smears, myeloid precursor cells increased to about 25% *of the granulocytic cell population The percentage* of lymphoid cells increased markedly but no erythroblasts were encountered (table II)

At 42 and 48 h after parabiosis, the cellularity of the bone marrow tissue increased still further Some younger erythroid precursor cells appeared in smears from some animals at 42 h after parabiosis An abrupt increase of erythroblasts was found at 48 h after parabiosis in irradiated animals Erythroblasts constituted more than 50% of the total nucleated cells and a marked decrease of lymphoid cells was present (table II)

Radioautography of bone marrow cells of the irradiated member collected 6 h after parabiosis and incubated with ^{3}HUr *in vitro*, revealed


a
b
c
d
e
f
g

Fig 1 Cells found in the aplastic bone marrow of an irradiated rat after parabiosis with a healthy litter mate *a* Metamyelocyte found 6 h after parabiosis *b* Myelocyte found 12 h after parabiosis *c* Promyelocyte found 18 h after parabiosis *d* Two myeloblasts found 24 h after parabiosis *e* Uptake of ^{3}HUr by mature granulocytes 6 h after parabiosis *f* Uptake of ^{3}HTdr by a myelocyte 12 h after parabiosis *g* Peroxidase reaction of mature granulocytes myelocytes and promyelocytes

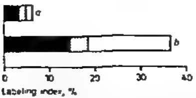


Fig 2 Mean labeling index of bone marrow cells by ³HTdr of irradiated (a) and nonirradiated (b) parabionts 18 h after parabiosis The labeling index was determined in each animal by counting 5 000 bone marrow cells. Dark area = myeloid cells, hatched area = lymphoid cells, open area = labeled cells other than myeloid and lymphoid cells

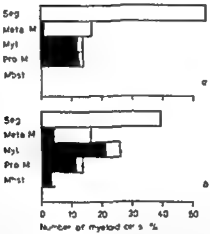


Fig 3 ³HTdr labeling index of myeloid cells of irradiated (a) and nonirradiated (b) parabionts at 18 h after parabiosis. Labeling index was obtained by counting 2,000 myeloid cells from bone marrow in each animal Seg = mature granulocytes, Meta M = metamyelocyte, Myl = myelocyte, Pro M = promyelocyte, Mbst = myeloblast open area = nonlabeled cells dark area = labeled cells.

RNA synthesis in large-sized granulocytes and metamyelocytes (fig 1e) These cells showed nuclear RNA synthesis without DNA synthesis Active RNA and DNA syntheses were observed in myelocytes found in the aplastic bone marrow 12 h after parabiosis (fig 1f) and also in promyelocytes at 18 h after parabiosis

In the *in vitro* study of DNA synthesis at 18 h after parabiosis, the labeling index of bone marrow cells was 6.3% and that of myeloid cell was

3.7%. In untreated parabionts labeling index of bone marrow cells was about 36% and that of myeloid cells was 13.7% (fig. 2). In irradiated animals, nearly 95% of all the precursor cells, myeloblasts, promyelocytes, and myelocytes, were found to be labeled (fig. 3a), while in those of the nonirradiated partner only 70% of myeloid precursors were labeled (fig. 3b). The labeling index of lymphoid cells was rather low compared to myeloid cells (fig. 2), although some large sized lymphoid cells were highly labeled. A similar tendency was observed in the labeling of lymphoid cells of the untreated partner.

In irradiated animals conjugated with partners having mature granulocytes labeled with ^{3}HTdr and sacrificed 18 h after parabiosis, the irradiated bone marrow contained labeled myeloid precursor cells and lymphoid cells, indicating that these cells migrated from the healthy partner.

Observations of peripheral blood smears at various time periods after parabiosis revealed no leukemoid reaction suggestive of a massive transfer of myeloid precursor cells from the healthy member. No myeloblasts or promyelocytes were found.

Discussion

Aplastic bone marrow of irradiated rats started granulopoiesis 24 h after parabiosis with a healthy littermate. Myelocytes, promyelocytes, and a few myeloblasts were observed 18 h after parabiosis, but mitosis was hardly encountered in these cells. At 24 h after parabiosis some myeloblasts in active mitosis appeared. Erythropoiesis appeared 42–48 h after parabiosis. Investigations with labeled cells and by chromosome analysis revealed that the cells participating in hemopoiesis in the irradiated bone marrow originated from the healthy partner.

The hemopoietic recovery of the aplastic bone marrow may have been directed by stem cells in circulating blood. In observations of colony-forming units (CFUs), McCULLOCH and TILL [12] noted that the cell number in any one colony showed an exponential increase with a 24 hour lag phase after transplantation, suggesting that mitosis in CFUs begins at 24 h after transplantation. This 24-hour lag phase is consistent with the granulopoiesis in the irradiated animal joined with a healthy partner. However, the precedency by erythropoiesis followed by granulopoiesis in CFUs [1] is inconsistent with the case.

The hemopoietic response becomes evident by the initial appearance of undifferentiated cells or the youngest precursor cells, followed by more

differentiated cells with mitosis In the present experiment, the erythropoiesis seen after parabiosis is consistent with the contemporary concept of hemopoiesis involving the stem cell of CFUs The erythropoiesis was triggered 42–48 h after parabiosis with the sudden appearance of the youngest precursor cells, followed by an abrupt increase of more differentiated erythroblasts

In myelopoiesis, however, the appearance of the youngest precursor cells was preceded by the appearance of more differentiated precursor cells This sequence differs from the process in erythropoiesis and is inconsistent with the general concept of hemopoiesis originating from the stem cell However, the participation of the stem cell or the transformation of lymphoid [13] or monocytic cells [14, 15] to myeloid precursor cells and the direct transformation of the stem cell to myeloblasts from its G_1 stage without differentiating mitosis should be considered [16]

In the present study a number of lymphoid cells appeared in the aplastic bone marrow after parabiosis, and large lymphocytes increased with time However, no cell was encountered that suggested a transformation of these cells to myeloid precursor cells, i e, myelocyte, promyelocyte, and myeloblast The myeloid precursor cells were clearly distinguished from lymphoid cells by a strong positive peroxidase reaction

Tyler and Everett [15] reported that in parabiotic rats, whose partner had ^{3}HTdr labeled cells by treating with ^{3}HTdr injections and temporary arrest of cross-circulation between 2 animals, some ^{3}HTdr labeled blast cells appeared in the bone marrow of the untreated animal 24 h after the last injection of ^{3}HTdr The blast cells demonstrated in their paper were very similar to those classified as myeloblasts and promyelocytes in this paper They supposed that monocytoid cells may give rise to these blast cells but we could not find any morphologic evidence to the direct transition of monocyte to these myeloid precursor cells They had distinct promyelocyte granules and gave strong positive peroxidase reaction, while monocyte had only faint azurophil granules and were slight or negative in peroxidase reaction

Another possibility is the direct transfer of myeloid precursor cells from the healthy partner to the irradiated animal The buffy coat smears of peripheral blood were examined carefully but neither promyelocytes nor myeloblasts were detected after parabiosis Tissue sections of liver, spleen and lymph nodes were observed but neither changes suggestive of leukemoid reaction nor accumulations of myeloid precursor cells were detected [17] The leukemoid reaction is generally accompanied by the mo-

bilization of erythroblasts, but in the present experiment erythroid cells were hardly encountered in smears of circulating blood. Furthermore, no erythroblast was found in the bone marrow of irradiated animals within 36 h after parabiosis. Almost all myeloid precursor cells in irradiated animals were at the S phase, while in the bone marrow of healthy partners many precursor cells were at the G_1 and G_2 stages. These observations are contrary to the direct transfer of the myeloid precursor cells from the healthy partner to the irradiated animal.

The blast formation of mature granulocytes has not been reported, but a possible explanation for the phenomenon observed in the present experiment appears to be the dedifferentiation of the granulocytes to myeloid precursor cells *in situ* and their participation in a new myelopoiesis in the aplastic bone marrow. Evidence for this possibility includes: accumulation of granulocytes in the irradiated bone marrow 3 h after parabiosis, RNA synthesis of some granulocytes at 6 h, appearance of myelocytes at 12 h, presence of promyelocytes with active RNA and DNA syntheses but without mitosis at 18 h, and the appearance of some myeloblasts with active mitosis at 24 h after parabiosis.

As reported in *in vitro* studies, the metamyelocyte may transform to myelocyte with a change in nuclear form [18]. Myelocytes seem to have a self-renewing activity [19]. Accordingly, metamyelocytes may induce granulopoiesis. In the present experiment, however, some cells having segmented nuclei showed RNA synthesis and this was followed by the appearance of myelocytes in the irradiated bone marrow. Therefore, it is not unconceivable that some granulocytes may rejuvenate to myelocytes.

In the present experiment, mitosis first appeared at the stage where myeloblasts were observed, and was hardly observed at the time when myelocytes and promyelocytes appeared. Therefore it appears that myelocytes have a self renewing activity but after dedifferentiation to myeloblast. Thus, in bone marrow some granulocyte may have the potency to rejuvenate to blast cell like as lymphocytes and participate in granulopoiesis under a condition such as severe hemopoietic tissue damage by lethal irradiation, although confirmation will require further investigations.

References

1 METCALF, D. and MOORE, M. A. S.: Haemopoietic cells. Front. Biol. 24: 70 (1971).

2 TILL, J. E. and MCCULLOCH, E. A.: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14: 213 (1961).

3 BARNES, D W H and LOUTIT, J F. Haemopoietic stem cells in the peripheral blood Lancet *II* 1138 (1967)
4 GOODMAN J W and HODGSON G S Evidence for stem cells in the peripheral blood of mice Blood *19* 702 (1962)
5 BECKER, A. J, MCCULLOCH, E. A., and TILL, J E. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature, Lond *197* 452 (1963)
6 BARNES D W H, EVANS, E P FORD, C. E., and WEST, B J Spleen colonies in mice Karyotypic evidence of multiple colonies from single cells. Nature, Lond. *219* 519 (1968)
7 PLUZNIK, D H and SACHS, L. The cloning of normal 'mast' cells in tissue culture J cell comp Physiol *66* 319 (1965)
8 BRADLEY, T R and METCALF, D The growth of mouse bone marrow cells *in vitro* Aust. J exp Biol med Sci *44* 287 (1966)
9 FANO C. H Improvement of the method of rats parabiosis with aortic anastomoses Acta Med Okayama *25* 597 (1971)
10 FANO C. H HIMEI, S., and SENO, S A new method of parabiosis by aortic anastomoses Transplantation *19* 354 (1975)
11 OMURA T A method of chromosome preparation for fresh bone marrow cells of the mouse Biol J Okayama Univ *16* 29 (1970)
12 MCCULLOCH E. A and TILL, J E. Proliferation of haemopoietic colony forming cells transplanted into irradiated mice. Radiat. Res. 22 383 (1964)
13 BEKKUM D W VAN, NOORD, M J VAN MAAT B., and DICKE, K. A Attempt at identification of hemopoietic stem cells in mouse Blood *38* 547 (1971)
14 TYLER R. W C. and EVERETT N B Radioautographic study of hemopoietic repopulation using irradiated parabiotic rats relation to stem cell problem Blood *28* 873 (1966)
15 TYLER R W and EVERETT N B Radioautographic study of cellular migration using parabiotic rats Blood *39* 249 (1972)
16 LAJTHA, L. G Cytokinetics and regulation of progenitor cells. J cell. comp Physiol *67* suppl 1 p 133 (1966)
17 HIRATA HIBI, M FANO C. H HIMEI, S NAKASHIMA, Y, HSUEH C. L., and SENO, S Morphologic studies on hemopoietic environment. Bone marrow, spleen, thymus and lymph nodes of the lethally irradiated rat after parabiosis by aortic anastomoses with healthy animal (in preparation)
18 PISCIOTTA A V and BRODY J L. Myelopoiesis *in vitro* Semin Hemat. *5* 275 (1968)
19 CARTWRIGHT G E. ATHENS, J W., and WINTROBE, M M. The kinetics of granulopoiesis in normal man Blood *24* 780 (1964)

Dr SATIMARU SENO, Department of Pathology Okayama University Medical School, *Okayama* (Japan)

Acta haemat 55 332–337 (1976)

Reconstitution Capacity of Bone Marrow Cells from Nude Mice in Radiation Chimeras

T W TAO and G L FLOERSHEIM

Laboratory for Transplantation Biology, Kantonsspital, Basel

Key Words Bone marrow reconstitution · Nude mice · Radiation chimeras · Transplantation

Abstract Lethally irradiated mice were reconstituted with bone marrow cells from 'nude' or normal mice It was found that the radioprotective capacity of bone marrow cells from normal and thymusless 'nude' mice is similar and that the immune responses to sheep red cells in radiation chimeras reconstituted with normal and nude bone marrow cells are also similar Bone marrow cells from nude mice in spite of the lack of the thymus develop into hematopoietic cells and contain also T precursor cells

Stem cells from the bone marrow are able to develop into cells of the erythroid, granulocytic and lymphoid series following transplantation into recipients receiving potentially lethal dose X-irradiation to result in radiation chimeras [4, 6, 8, 11] It is not clear how this development is controlled One of the controlling mechanisms postulated is the thymus The role of thymus in the development of the lymphoid system is well documented [2] and its role in hematopoiesis has also been suggested [3, 10] ZIPORI and TRAININ [17] have reported that the radioprotective capacity of the bone marrow cells from neonatally thymectomized mice was greatly impaired and that bone marrow cells from nude mice (nu/nu) which lack the thymus congenitally, only occasionally exhibit radioprotection [17] This paper describes experiments showing that (a) the radioprotective capacity of bone marrow cells from normal and nude mice is similar and that (b) the immune responses to sheep red cells in radiation chimeras reconstituted with normal and nude bone marrow cells are also similar

Material and Methods

Normal C3H and C57Bl 6 mice and homozygous nu nu mice backcrossed (4th–5th backcross) with C3H and C57Bl 6 backgrounds were used (purchased from Gl Bomholtgard Ltd., Ry, Denmark) Donor and recipient animals were 8–10 weeks old Bone marrow cells were prepared in cold from tibiae and femora in Eagle s minimal essential medium supplemented with 10% fetal calf serum. Donor cells were pooled and injected intravenously at the indicated dosages within 2–3 h following irradiation Cell doses per mouse ranging from 1×10^7 to 1×10^5 from normal and nude mice were tested for their ability to save lethally irradiated recipients. 10 to 20 mice were used for each group Animals were kept on antibiotics (100 mg Neomycin and 10 mg Polymyxin/liter added in drinking water) for 2 weeks and observed daily for mortality for 3–4 months.

The recipients were prepared for the bone marrow graft by exposure to 850 r of total body irradiation given at a rate of 35 r/min and a distance of 25 cm by a Philips RT 305 X ray machine [300 kV, 10 mA, total filtration 2 7 mm (Cu)]

Results

Figure 1 shows that the mortality rates of lethally irradiated C3H mice reconstituted with varying doses of bone marrow from nude C3H mice and from normal C3H mice were similar With 1×10^7 bone marrow cells there was no mortality With 3×10^5 bone marrow cells the mortality was 23%, whereas with 1×10^5 cells it was 50%. All control mice which received no bone marrow cells died within 2 weeks Figure 2 shows the mortality curve with C57Bl/6 mice With bone marrow cell doses of 1×10^7, 3×10^4 and 1×10^5 cells, mortality amounted to 21, 38 and 61%, respectively, for those reconstituted with normal C57BL6 bone marrow cells, and to 30, 16 and 84% for those reconstituted with nude C57BL6 bone marrow cells All controls which were only irradiated died within 2 weeks The mortality rates of mice restored with equal cell doses from either nude or normal donors were not significantly different (Fisher s exact test) The results with both mouse strains show the equivalent potency of bone marrow cells from nude and normal mice to rescue irradiated mice by restoring their hematopoiesis

To test the reconstitution of the immunological capacity of the surviving mice, sheep red blood cells (SRBC) were chosen as the antigen because the immune response to this antigen in the normal [13] and nude [15] mice is well documented It is classified as a T-cell (thymus cell) dependent antigen The immune response in the nudes is primarily of the IgM type A single injection of 0.5 ml of 10% sheep red cells was given

Acta haemat. 55: 332–337 (1976)

Reconstitution Capacity of Bone Marrow Cells from Nude Mice in Radiation Chimeras

T. W. Tao and G. L. Floersheim

Laboratory for Transplantation Biology, Kantonsspital Basel

Key Words. Bone marrow reconstitution · Nude mice · Radiation chimeras · Transplantation

Abstract. Lethally irradiated mice were reconstituted with bone marrow cells from 'nude' or normal mice. It was found that the radioprotective capacity of bone marrow cells from normal and thymusless 'nude' mice is similar and that the immune responses to sheep red cells in radiation chimeras reconstituted with normal and nude bone marrow cells are also similar. *Bone marrow cells from nude mice, in spite of the lack of the thymus, develop into hematopoietic cells and contain also T precursor cells.*

Stem cells from the bone marrow are able to develop into cells of the erythroid, granulocytic and lymphoid series following transplantation into recipients receiving potentially lethal dose X-irradiation to result in radiation chimeras [4, 6, 8, 11]. It is not clear how this development is controlled. One of the controlling mechanisms postulated is the thymus. The role of thymus in the development of the lymphoid system is well documented [2] and its role in hematopoiesis has also been suggested [3, 10]. Zipori and Trainin [17] have reported that the radioprotective capacity of the bone marrow cells from neonatally thymectomized mice was greatly impaired and that bone marrow cells from nude mice (nu/nu), which lack the thymus congenitally, only occasionally exhibit radioprotection [17]. This paper describes experiments showing that (a) the radioprotective capacity of bone marrow cells from normal and nude mice is similar and that (b) the immune responses to sheep red cells in radiation chimeras reconstituted with normal and nude bone marrow cells are also similar.

Material and Methods

Normal C3H and C57Bl/6 mice and homozygous nu/nu mice backcrossed (4th–5th backcross) with C3H and C57Bl 6 backgrounds were used (purchased from Gl Bomholtgard Ltd., Ry Denmark) Donor and recipient animals were 8–10 weeks old. Bone marrow cells were prepared in cold from tibiae and femora in Eagle s minimal essential medium supplemented with 10% fetal calf serum. Donor cells were pooled and injected intravenously at the indicated dosages within 2–3 h following irradiation. Cell doses per mouse ranging from 1×10^{7} to 1×10^{5} from normal and nude mice were tested for their ability to save lethally irradiated recipients 10 to 20 mice were used for each group Animals were kept on antibiotics (100 mg Neomycin and 10 mg Polymyxin/liter added in drinking water) for 2 weeks and observed daily for mortality for 3–4 months

The recipients were prepared for the bone marrow graft by exposure to 850 r of total body irradiation given at a rate of 35 r/min and a distance of 55 cm by a Philips RT 305 X ray machine (300 kV 10 mA total filtration 2 7 mm (Cu))

Results

Figure 1 shows that the mortality rates of lethally irradiated C3H mice reconstituted with varying doses of bone marrow from nude C3H mice and from normal C3H mice were similar With 1×10^{7} bone marrow cells there was no mortality With 3×10^{5} bone marrow cells the mortality was 23%, whereas with 1×10^{5} cells it was 50%. All control mice which received no bone marrow cells died within 2 weeks Figure 2 shows the mortality curve with C57Bl/6 mice With bone marrow cell doses of 1×10^{7}, 3×10^{6} and 1×10^{5} cells, mortality amounted to 21, 38 and 61%, respectively, for those reconstituted with normal C57Bl 6 bone marrow cells and to 30, 16 and 84% for those reconstituted with nude C57Bl 6 bone marrow cells All controls which were only irradiated died within 2 weeks The mortality rates of mice restored with equal cell doses from either nude or normal donors were not significantly different (Fisher s exact test) The results with both mouse strains show the equivalent potency of bone marrow cells from nude and normal mice to rescue irradiated mice by restoring their hematopoiesis

To test the reconstitution of the immunological capacity of the surviving mice, sheep red blood cells (SRBC) were chosen as the antigen, because the immune response to this antigen in the normal [13] and nude [15] mice is well documented It is classified as a T-cell (thymus cell) dependent antigen The immune response in the nudes is primarily of the IgM type A single injection of 0 5 ml of 10% sheep red cells was given

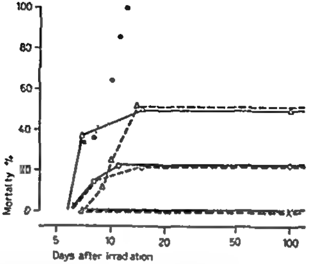


Fig 1 Mortality of 850 r irradiated C3H mice transplanted with varying doses of bone marrow cells from normal (———) and nude (- - -) C3H mice ● = Controls, no cells Δ = 1 × 10⁵ cells ○ = 3 × 10⁵ cells × = 1 × 10⁷ cells

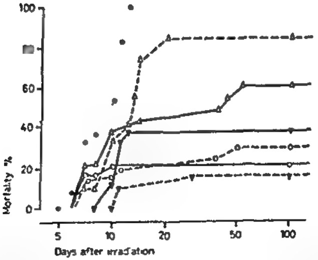


Fig 2 Mortality of 850 r irradiated C57Bl/6 mice transplanted with varying doses of *bone marrow cells from normal (———) and nude (- - -)* C57Bl/6 mice ● = Controls, no cells. Δ = 1 × 10⁵ cells. ▼ = 3 × 10⁶ cells ○ = 1 × 10⁷ cells

Table I Immune response to SRBC in radiation chimeras produced by normal and nude C3H bone marrow cells transplanted into lethally irradiated C3H mice

Bone marrow cells transplanted		PFC/10⁶ spleen cells	
donor	cell dose per mouse	direct	indirect
nu/nu	3×10^{5}	154 ± 42	892 ± 284
	1×10^{7}	83 ± 19	615 ± 216
Normal	3×10^{5}	139 ± 30	795 ± 90
	1×10^{7}	119 ± 37	577 ± 122

Hemolytic plaque forming spleen cells (PFC) were assayed 7 days after intravenous injection of SRBC and 4 months after bone marrow transplantation. Mean values ± SE for groups of 4–6 mice are given. The respective differences of the PFCs after nu/nu and normal bone marrow cells are not statistically significant

intravenously or intraperitoneally to the radiation chimeras 2–4 months after bone marrow transplantation. The spleen cells were assayed 7 days later for cells producing IgM antibodies (direct plaques) and IgG antibodies (indirect plaques) according to the CUNNINGHAM and SZENBERG [1] modification of the hemolytic plaque assay [5]. Table I shows that the number of cells producing antibodies against sheep red cells in irradiated C3H mice reconstituted with nude and normal C3H bone marrow cells 4 months earlier was similar. There were both IgM and IgG antibody producers, with more of the latter type, similar to that found in the normal mice. This means that their immunological capacity, at least to this antigen, was probably fully reconstituted. Table II shows a similar result from irradiated C57Bl/6 mice 2 months after bone marrow transplantation.

Discussion

The results reported here imply that there is no defect exhibited by the bone marrow cell populations in the nude mice with radioprotection and immune reactivity as indices, in spite of the lack of the thymus. These cells transplanted into lethally irradiated mice were able to develop not only into hematopoietic cells as shown by their ability to save lethally irradiated mice, but also into lymphoid cells including thymus cells as shown by the unimpaired ability to produce both IgM and IgG responses to a 'thymus-dependent' antigen. One could always raise the question

Table II. Immune response to SRBC in radiation chimeras produced by normal and nude C57Bl/6 bone marrow cells transplanted into lethally irradiated C57Bl 6 mice

Bone marrow cells transplanted		PFC/10^6 spleen cells	
donor	cell dose per mouse	direct	indirect
nu/nu	1×10^7	475 ± 14	>2 000
Normal	1×10^7	533 ± 81	~2 000

PFC were assayed 7 days after intraperitoneal injection of SRBC and 2 months after bone marrow transplantation. Mean values ± SE for groups of 5 mice are given. The differences are not statistically significant.

whether these results are real in that the nude bone marrow cells did indeed develop normally as normal bone marrow cells following transplantation into lethally irradiated recipients or are deceptive in that the irradiated hosts recovered from the irradiation effects. The second possibility is rendered unlikely by experiments showing that the hematopoietic and lymphoid cells in radiation chimeras produced in this way are predominantly of the donor instead of the recipient type [6, 9, 12]. If this is the case, the T-cells in the mice reconstituted with nude bone marrow cells would have to come from these transplanted cells. In other words, there are T-precursor cells in the nude bone marrow as observed by other investigators [9, 14]. LOOR and KINDRED [7] have also come to the same conclusion by studying the T-cell membrane markers following grafting neonatal AKR thymus (TL⁻, θ AKR) into Balb/C nude mice (TL2, θ C3H). Experiments are under way using allotype markers to study the possible switch-over of the recipient allotype to donor allotype in these radiation chimeras reconstituted with bone marrow cells from the nude mice.

Acknowledgements. We thank Dr. BRUCE ELLIOTT for helping with a part of the plaque assay and Mrs. M. CERESHETTI for assistance. This work was supported by the Swiss National Foundation for Medical Research.

References

1 CUNNINGHAM, A. J. and SZENBERG, A.: Further improvements in the plaque technique for detecting single antibody forming cells. Immunology 14: 599 (1968).

2 DEFENDI, V. and METCALF, D. (ed.) The thymus. (Wistar Inst. Press, Philadelphia 1964).

3 GOODMAN, J. W. and GRUBBS, C. G.: The relationship of the thymus to erythropoiesis, in STOHLMAN Hemopoietic cellular proliferation, p. 26 (Grune & Stratton, New York 1970).

4 ILBERY, P. L. T., KOLLER, P. C., and LOUTIT, J. F.: Immunological characteristics of radiation chimeras. J. natn. Cancer Inst. *20*: 1051 (1968).

5 JERNE, N. K., NORDIN, A. A., and HENRY, D.: The agar plaque technique for recognizing antibody producing cells, in AMOS and KOPROWSKI Cell bound antibodies, p. 109 (Wistar Press, Philadelphia 1963).

6 KOLLER, P. C., DAVIES, A. J. S., and DOAK, M. A.: Radiation chimeras. Adv. Cancer Res. 6: 181 (1961).

7 LOOR, F. and KINDRED, B.: Differentiation of T-cell precursors in nude mice demonstrated by immunofluorescence of T-cell membrane markers. J. exp. Med. *138*: 1044 (1973).

8 LORENZ, E., UPHOFF, D. E., REID, T. R., and SHELTON, E.: Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. natn. Cancer Inst. *12*: 197 (1951).

9 PRITCHARD, H. and MICKLEM, H. S.: Haemopoietic stem cells and progenitors of functional T lymphocytes in the bone marrow of 'nude' mice. Clin. exp. Immunol. *14*: 597 (1973).

10 RESNITZKY, P., ZIPORI, D., and TRAININ, N.: Effect of neonatal thymectomy on hemopoietic tissue in mice. Blood *37*: 634 (1971).

11 TRENTIN, J. J.: Grafted marrow rejection mortality contrasted to homologous diseases in irradiated mice receiving homologous bone marrow. J. natn. Cancer Inst. 22: 219 (1959).

12 WALLIS, V. J., LEUCHARS, E., CHWALINSKI, S., and DAVIES, A. J. S.: On the sparse seeding of bone marrow and thymus in radiation chimeras. Transplantation *19*: 2 (1975).

13 WORTIS, H. H., TAYLOR, R. B., and DRESSER, D. W.: Antibody production studied by means of the LHG assay. I. The splenic response of CBA mice to sheep erythrocytes. Immunology *11*: 603 (1966).

14 WORTIS, H. H., NEHLSEN, S., and OWEN, J. J.: Abnormal development of the thymus in 'nude' mice. J. exp. Med. *134*: 681 (1971).

15 WORTIS, H. H.: Immunological studies of nude mice, in COOPER and WARNER Contemporary topics in immunobiology, vol. 3, p. 243 (Plenum Press, New York 1974).

16 ZIPORI, D. and TRAININ, N.: Defective capacity of bone marrow from nude mice to restore lethally irradiated recipients. Blood *42*: 671 (1973).

17 ZIPORI, D. and TRAININ, N.: Impaired radioprotective capacity and reduced proliferative rate of bone marrow from neonatally thymectomized mice. Exp. Hemat. 3: 1 (1975).

Prof. G. L. FLOERSHEIM, Laboratory for Transplantation Biology, Kantonsspital, Petersgraben 17, *CH-4056 Basel* (Switzerland)

Acta haemat. 55: 339–345 (1976)

Characterization of Blast Cells in Acute Nonlymphoid Leukemias by Consecutive Cytochemical Reactions

With 1 color plate

G. D. GRUSOVIN and G. L. CASTOLDI

Department of Internal Medicine, University of Ferrara, Ferrara

Key Words. Acute leukemia · Blast cells · Combined cytochemical stainings · Cytochemistry · Esterases · Leukemia

Abstract. Blast cells of 12 acute nonlymphoid leukemias have been investigated by consecutive application on the same preparation of three cytochemical reactions: α-naphthylacetate esterase, AS-D chloroacetate esterase and PAS. A fourth reaction (α-naphthylbutyrate esterase) was added in some preparations for a more detailed definition of the monocytoid oriented cells. The cytochemical techniques were further combined with NaF inhibition and with the cytobacterial test for muramidase. The combined cytochemical approach allowed the identification of most of the blast cells, according to an empirically determined positivity to one or more reactions, and proved to be particularly valuable in classifying the cells after chemotherapy.

The variable responsiveness of acute leukemias to chemotherapy has supported further attempts to classify different cytological variants with particular prognostic properties. In this regard several approaches have been made recently with reference to the pictures in Romanovsky preparations [18, 19], as well as to immunocytological properties [1, 6, 17], cytochemical features [2, 11, 20], ultrastructural aspects [3], and some functional characteristics [7, 23].

While the acute lymphoblastic leukemias have been recently characterized more accurately on the basis of the immunocytological markers, much more difficulties have occurred in the definition of the acute nonlymphoid leukemias which apparently show a minor homogeneity. Two major problems have been encountered in the classification of the blast cells in the nonlymphoid acute leukemias: (1) the search for suitable markers of the myeloid cell series, (2) the possibility to identify the differen-

tiation of the myeloid cells, with particular reference to the monocytoid elements [26]. These difficulties are explaining the requirement of additional tests in order to achieve a more complete evaluation of the activity of the blastic elements [7, 12, 23].

Since most of the therapeutic managements are performed accordingly to the criteria of the bone marrow composition and the peripheral blood picture, the morphological approach appears still to be the most valuable tool for diagnostic purposes [10].

A series of cytochemical and cytofunctional tests consecutively applied on the same slide has provided the possibility to classify most of the blast cells and of their differentiation. This consecutive staining procedure proved to be particularly useful in our hands for the characterization of the blastic population during the transformation of the chronic myeloid leukemia [5].

Material and Methods

12 patients of both sexes, aged between [illegible] and 74 years, with acute nonlymphoid leukemias were examined before treatment and during the course of the illness for a follow up of the blastic cell population. For this purpose three cytochemical reactions were consecutively performed on the blood smears: α-naphthylacetate esterase (α-NAE) [15], AS-D-chloroacetate esterase (AS-D-Ch AE) [13] and PAS. A fourth reaction, the α-naphthylbutyrate esterase (α-NBE) [14], was added. The use of the α-NBE provided more reliability to the definition of the monocytoid component of the population, since the substrate employed in this reaction has been ac

AS-D Ch AE AS-D Ch AE + PAS (granular)		→ myeloblasts
AS-D-Ch AE + α-NAE (or α-NBE)		→ promonocytes
α-NAE	+ PAS (strong and coarse irregular positivity)	→ megakaryoblasts
	+ PAS (diffuse or fine granular + NAE (inhibition) + lysozyme (production) α-NBE (substrate substitution)	monoblasts, monocytes

Fig. 1. Identification of blast cells by combined cytochemical reactions.

Table I Percentage of blast cells with positive reaction

	Case No											
	1	2	3	4	5	6	7	8	9	10	11	12
Promyelocytes, myeloblasts AS-D-Ch-AE AS-D-Ch-AE+PAS	91	63	60	7	79	40	62	42	74	57	41	68
Promonocytes AS-D-Ch-AE+α-NAE AS-D Ch-AE+α-NBE	5	14	19	–	8	23	15	11	5	8	16	6
Megakaryoblasts α-NAE α-NAE+PAS	–	–	1	–	2	–	–	1	–	3	–	–
Monoblasts, monocytes α-NBE α-NBE+PAS	4	23	20	11	11	37	21	46	21	32	43	24
No reaction	–	–	–	82	–	–	2	–	–	–	–	–

tually demonstrated to be strongly specific for the monocytic series [14] The combined reactions allowed the differentiation of most blast cells, according to an empirically determined positivity to one or more reactions (fig 1)

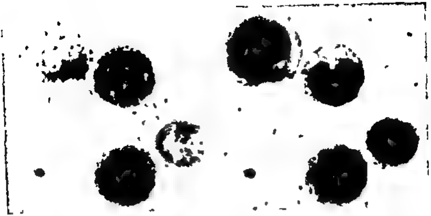
500 cells with blastic features were scored on each preparation and a percent distribution, according to the previously defined cytochemical characteristics, was reported

The definition of the mononuclear elements (monocytic) has been further investigated by means of the cytobacterial test for the muramidase production [25] and the inhibition test with (NaF) [22], applied together with the multiple cytochemical stainings on the same slide

Some preparations were finally decolorized in xylene with removal of the cytochemical staining, and subsequently restained with May-Grünwald Giemsa in order to obtain a comparison between the usual panoptic features of the cells and the cytochemical properties demonstrated by the consecutive stainings

Results

The results of the distribution of the blast cells according to the combined cytochemical features are listed in table I. Apart from an unclassifiable case only a small proportion of the blastic cells was not identified Most of the cases were mixed forms with a variable participation of granuloblastic and monocytoid cells. Pure myeloblastic forms (more than 80%



a b

Fig. 2. *a* Combined cytochemical reactions for AS-D-Cb-AE and α-NBE. Myeloblastic elements present the cytoplasm filled with dark granules (fast violet LB salt), while the monocytoid cells show coarser granules (fast garnet). *b* Same picture after decoloration and restaining with May-Grünwald-Giemsa. Two cells with similar shape and indented nuclei appear to be differently classified on the basis of the combined cytochemical procedures (case No. 8). × 670.

of the elements) represented only a minor fraction of our cases 1 case out of 12 was unclassifiable because of the lack of all the markers in most of the cells (case No 4) This case possibly could be interpreted as a poorly differentiated leukemia

Monocytoid elements were clearly identified with the reaction for the α-NBE The usefulness of this reaction in association with the AS-D Ch-AE could be further evaluated by reviewing the cells after elution of the cytochemical stainings and restaining with May-Grunwald-Giemsa In fact mononuclear cells with indented nuclei which appear quite similar in Romanovsky preparations, could be easily identified as belonging to different lines (granulocytic and monocytic, respectively) in the cytochemically treated smears (fig 2, 3)

The introduction of the cytobacterial test in association with the cytochemical reactions allowed a finer distinction of the monocytoid elements with regard to their degree of maturation Monoblastic cells were characterized by a weak α-NAE activity (eventually associated with a fine granular PAS positivity) and by a poor muramidase secreting activity, in contrast with the more nature monocytic cells which showed usually a pronounced muramidase production (fig 4) Inhibition with NaF could further confirm the nature of these elements

An interesting observation was the appearance in a few cases of elements with the features of megakaryoblasts well defined by a strongly positive α-NAE reaction (sometimes associated with coarse PAS-positive granules surrounding the cytoplasm) and by an almost completely negative reaction for the α-NBE These elements, which are very common in the blastic crisis of the chronic myeloid leukemia were demonstrable only in a minority of the cases of acute leukemia and in a small percentage

Discussion

On the basis of the cytochemical distribution of the blast cells in our cases of acute nonlymphoid leukemias the following types are likely to be recognized myeloblastic, promyelocytic, promonocytic, myelomonocytic, monocytic, and unclassifiable This distinction is in agreement with the functional differentiation of the blast cells [7–9] Particularly, similar results have been obtained by BENNETT and REED [2] utilizing an empirically determined positivity to a series of different reactions (peroxidase, PAS NASDA and NASDA + F)

a b

Fig. 3. *a* Combined AS D Ch AE and *α* NBE (case No. 12). *b* Combined AS D Ch AE + *α* NBE + PAS (case No. 12). Monocytoid cells may show, besides the positive reaction for the *α* NBE, several fine PAS positive granules scattered over the cytoplasm. ×1,000.

In our opinion the most outstanding aspect of the consecutive application of the cytochemical reactions seems to be represented by a more detailed definition of the monocytoid-oriented cells and possibly, by the differentiation between the monoblastic and the more mature monocytic elements. The possibility to identify the megakaryoblastic elements may be another important point, since small megakaryoblasts have been recognized only by means of multiple combined approaches, including the ultrastructural procedures [16, 24] in several conditions of preleukemia and in a variety of myeloproliferative disorders [16]. With the combined cytochemical stainings they were easily recognized in a significant number in the blastic crisis of various cases of chronic myeloid leukemia where they may represent a great part of the blast cell population [4]. In acute nonlymphoid leukemias the megakaryoblasts did not represent a significant component of the blast cell population, however, we found a consistent proportion of these cells in one case which had a transient erythremic picture during the course of the illness. A small number of megakaryoblasts may not be uncommon in mixed forms of acute leukemia (myelomonocyt-

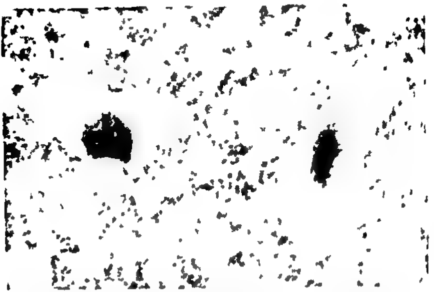


Fig. 4. Combined preparation for AS-D Ch-AE + α-NBE + FAS + cytochemical test for lysozyme activity. A large area of lysis is shown around an α-NAE positive cell (most likely monocyte, left). A pale decoloration area is present around a cell (right) with the features of a promonocyte (positive reaction for both the AS-D Ch-AE and the α-NAE) × 1,200.

ic) or in a terminal phase of the illness, when the blood picture is often recalling the features of a panmyelopathy. In this respect Saarni and Linman [21] have already emphasized that the myelomonocytic forms of leukemia may represent a global involvement of the bone marrow.

The destaining of the preparations provides additional information on the nature of the cells and allows a comparison with the panoptically stained preparates. A misleading interpretation of the Giemsa features is particularly prominent after chemotherapy which alters significantly the morphology of the cells. The characterization of the cells by consecutive cytochemical stainings and the observation of the Giemsa features of the same elements after destaining appear highly valuable for diagnostic purposes and corroborate the opportunity of a double sequence in the cytological identification.

References

1 Belpaire, D.; Dantchev, D.; De Reuck, F.; Chamone, D.; Hecke, P.; Poortmans, P.; Schwarzenberg, L.; Amiel, J. L., and Mathé, G.: ...

19 MATHÉ, G., BELPOMME, D., DANTCHEV, D., POUILLART, P., NAVARES, L., HAUSS, G., SCHLUMBERGER, J. R., and LAFLEUR, M.: Search for correlations between cytological types and therapeutic sensitivity of acute leukemias. Blood Cells *1*: 37–52 (1975).

20 QUAGLINO, D.: Cytological and cytochemical aspects of acute leukemias; in MANDELLI, AMADORI and MARIANI Therapy of acute leukemias. Proc. Int. Meet., Rome 1973, pp. 17–58 (Minerva Medica, Torino 1974).

21 SAARNI, M. I. and LINMAN, J. W.: Myelomonocytic leukemia: disorderly proliferation of all marrow cells. Cancer *27*: 1221–1230 (1971).

22 SCHMALZL, F. und BRAUNSTEINER, H.: Cytochemische Darstellung von Esterase-Aktivitäten in Blut- und Knochenmarkszellen. Klin. Wschr. *46*: 643–650 (1968).

23 SCHMALZL, F., HUHN, D., ASAMER, H., and BRAUNSTEINER, H.: Hairy cell leukemia (leukemic reticuloendotheliosis?): reticulosarcoma and monocytic leukemia. Acta haemat. *53*: 257–276 (1975).

24 SMITH, W. B., ABLIN, A., GOODMAN, J. R., and BRECHER, G.: Atypical megakaryocytes in preleukemic phase of acute myeloid leukemia. Blood *42*: 535–540 (1973).

25 SYRÉN, E. and RAESTE, A. M.: Identification of blood monocytes by demonstration of lysozyme and peroxidase activity. Acta haemat. *45*: 29–35 (1971).

26 YAM, L. T., LI, C. Y., and CROSBY, W. H.: Cytochemical identification of monocytes and granulocytes. Am. J. clin. Path. *55*: 283–290 (1971).

Dr. G. D. GRUSOVIN and Dr. G. L. CASTOLDI, Clinica Medica Generale, Università di Ferrara, *I-44100 Ferrara* (Italy)

phocyte markers on the neoplastic cells of 20 patients with chronic lymphoid leukemias Biomedicine *20* 109–118 (1974)
2 BENNETT, J M and REED, C E Acute leukemia cytochemical profile diagnostic and clinical implications Blood Cells *1* 101–108 (1975)
3 BESSIS, M Cytologic diagnosis of leukemias by electron microscopy Rec Res Cancer Res *43* 63–70 (1973)
4 CASTOLDI G L und GRUSOVIN, G D Megakaryozyten und Monozytenformen im Blastenschub der chronischen myeloischen Leukamie, in STACHER Proliferative Erkrankungen des myeloischen Systems 3 Int Arbeitstagung Wien 1975 (in press)
5 CASTOLDI G L., GRUSOVIN, G D, and SCAPOLI, G L Consecutive cytochemical staining for the analysis of the blastic population in the acute phase of chronic myeloid leukemia Biomedicine *23* 12–16 (1975)
6 CATOVSKY, D, FRISCH, B, and VAN NOORDEN, S B, T and Null cell leukemias Electron cytochemistry and surface morphology Blood Cells *1* 115–124 (1975)
7 CATOVSKY, D, HOFFBRAND, A V, IKOKU, N B, PETRIE, A, and GALTON D A G Significance of cell differentiation in acute myeloid leukemia Blood Cells *1* 201–211 (1975)
8 FLANDRIN, G and BERNARD, J Cytological classification of acute leukemias A survey of 1400 cases Blood Cells *1* 7–15 (1975)
9 GALTON, D A G and DACIE, J V Classification of acute leukemias Blood Cells *1* 17–24 (1975)
10 GLICK, A D and HORN, R G Identification of promonocytes and monocytoid precursors in acute leukemia of adults ultrastructural and cytochemical observations *Br J Haemat 26 395–403 (1974)*
11 HAYHOE, F G J Leukemia and lymphoma the prospect of control Schweiz med Wschr *104* 1897–1907 (1974)
12 KARLE, H, HANSEN N E, and KILLMANN, S A *Intracellular lysozyme in mature neutrophils and blast cells in acute leukemia* Blood *44* 247–255 (1974)
13 LEDER, L D Der Blutmonocyt (Springer, Berlin 1967)
14 LI K. Y, LAM K W., and YAM L T Esterases in human leucocytes J Histochem Cytochem *21* 1–12 (1973)
15 LÖFFLER, H Cytochemischer Nachweis von unspezifischer Esterase in Ausstrichen Klin Wschr *39* 1220–1227 (1961)
16 MALDONADO, J E Dysplastic platelets and circulating megakaryocytes in chronic myeloproliferative diseases II Ultrastructure of circulating megakaryocytes Blood *43* 811–820 (1974)
17 MARMONT, A, DAMASIO E, SANTINI G, GIORDANO D, and BACIGALUPO, A An immunohistochemical approach to the cytological diagnosis of acute lymphoblastic leukemia in MANDELLI AMADORI and MARIANI Therapy of acute leukemias *Proc Int Meet, Rome 1973 pp 737–748 (Minerva Medica, Torino 1974)*
18 MATHE, G, POUILLART P, STERESCU, M, AMIEL, I, SCHWARZENBERG L., SCHNEIDER M, HAYAT, M, DE VASSAL, F, JASMIN, G et LAFLEUR, M Subdivision des variétés classiques des leucémies aigues corrélation avec prognostic et des chances de guérison Revue eur Etud clin biol *16* 554–560 (1971)

19 Mathé, G Belpomme D Dantchev D Pouillart P Navares L Hauss G Schlumberger J R., and Lafleur, M Search for correlations between cytological types and therapeutic sensitivity of acute leukemias Blood Cells 1 37 52 (1975)
20 Quaglino D Cytological and cytochemical aspects of acute leukemias in Mandelli Amadori and Mariani Therapy of acute leukemias Proc Int Meet., Rome 1973 pp 17 58 (Minerva Medica, Torino 1974)
21 Saarni M I and Linman J W Myelomonocytic leukemia disorderly proliferation of all marrow cells Cancer 27 1221 1230 (1971)
22 Schmalzl, F und Braunsteiner H Cytochemische Darstellung von Esterase Aktivitäten in Blut und Knochenmarkszellen Klin Wschr 46 643–650 (1968)
23 Schmalzl, F Huhn D Asamer H., and Braunsteiner, H Hairy cell leukemia (leukemic reticuloendotheliosis) reticulosarcoma and monocytic leukemia Acta haemat 53 257 276 (1975)
24 Smith W B Ablin A Goodman J R., and Brecher G Atypical megakaryocytes in preleukemic phase of acute myeloid leukemia Blood 42 535–540 (1973)
25 Syren E and Raeste, A M Identification of blood monocytes by demonstration of lysozyme and peroxidase activity Acta haemat 45 29 35 (1971)
26 Yam L T Li C. Y., and Crosby W H Cytochemical identification of monocytes and granulocytes. Am J clin Path. 55 283–290 (1971)

Dr G D Grusovin and Dr G L Castoldi Clinica Medica Generale Università di Ferrara I-44100 Ferrara (Italy)

Acta haemat 55 346–353 (1976)

Malignant Lymphoma with Plasmacytoid Differentiation and Polyclonal Gammopathy

J Guardia J D Pedreira M T Vidal and A Roca

Departments of Internal Medicine and Pathology Ciudad Sanitaria de la Seguridad Social Universidad Autónoma Barcelona

Key Words Immunoglobulins · Lymphoplasmacytoid proliferation · Malignant lymphoma · Plasma cells · Polyclonal gammopathy

Abstract Two patients are presented with lymphadenopathy hepatosplenomegaly and infiltration of the bone marrow with plasmocytoid cells in whom an important heterogeneous hyperimmunoglobulinemia was detected (3 18 and 5 85 g/100 ml) Autopsy showed widespread poorly differentiated lymphocytic lymphoma with plasmocytoid differentiation in the first case and a well differentiated lymphocytic lymphoma with plasmocytoid differentiation involving lymph nodes marrow spleen liver and kidney in the second case High concentrations of IgM and IgG were detected in lymph node and spleen homogenates from this case After splenectomy and cyclophosphamide the immunoglobulins decreased It is suggested that both cases had a malignant lymphoma with plasmocytoid differentiation and polyclonal gammopathy

A monoclonal paraprotein may be often seen in lymphoproliferative or plasmocytic disorders [7, 8] In nonneoplasic diseases such as hepatic cirrhosis or chronic infections a polyclonal increase of immunoglobulins is usual, but this is not a common feature of malignant lymphoma In 2 patients we recently observed a polyclonal gammopathy together with a widespread malignant proliferation with plasmocytoid differentiation

Case Reports

Case 1 M G G a 71 year old male who 2 months before entry complained of anorexia malaise and weight loss 40 days later a macular purpuric rash in the lower extremities and fever appeared Multiple lymph node enlargements were felt In

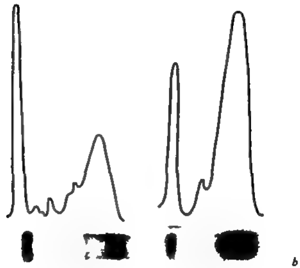
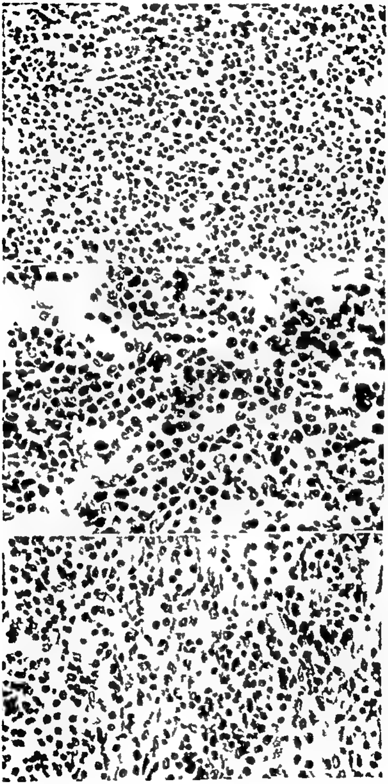


Fig 1 Serum (a) and urine (b) electrophoresis in case 1, showing a polyclonal increase of immunoglobulins confirmed by immunoelectrophoresis

the abdomen liver and spleen were felt 6 cm below the costal margin. Chest X rays showed left hiliary lymph nodes. The patient died 10 days later.

Laboratory data. Hematocrit 3?%, hemoglobin 11.9 g/100 ml, white cell count 16,800 with 11% band forms, 64% neutrophils, 21% lymphocytes and 4% monocytes; sedimentation rate 120 mm/h. Coombs test negative, total proteins 7.1 g/100 ml with 37.79% albumin, 1.57% α_1, 4.72% α_2, 11.02% β and 44.8% heterogeneous γ-globulins. Immunoelectrophoresis showed a polyclonal increase of all three major immunoglobulins. Free heavy chains were not detectable by monospecific antiserums and no M component could be detected in serum or urine. In the urine total proteins were 990 mg/24 h, with 17.5?% albumin, 0.5% α_1, 3.51% α_2, 5.02% β and 73.3?% γ globulins (fig. 1b). Bone marrow aspiration showed 72% of plasmocytic cells. Skull and spine X rays were normal.

Necropsy. There were multiple peripheral lymph node enlargements in thorax and abdomen. Their general structure was not preserved; there were multiple nodular areas formed by plasmocytoid and some lymphoid cells. The capsula was invaded and the most peripheral sinus preserved, but the central sinus had generally disappeared. The cells were pleomorphic, some very big, irregular and with prominent nucleolus, while others were small with dense nuclei and scanty protoplasm. There were also some plasma cells of normal aspect. The spleen was 400 g and invaded by the same cellular elements (fig. 2a). There was bilateral pleural and pericardial effusion. The lungs showed peribronchial and perivascular infiltrations of lymphoid and plasmocytoid cells and of some reticular cells. In the abdomen there was peritoneal effusion. The liver weighed 1,500 g and showed some plasmocytoid infiltrations of



a

b

c

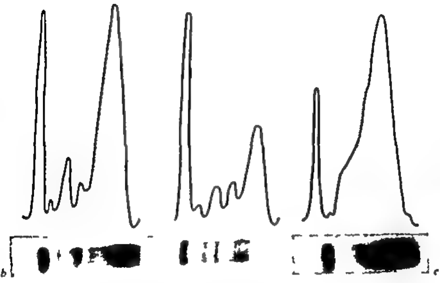


Fig 3 Serum electrophoresis in case 2 before (a) and 2 months after (b) splenectomy and cyclophosphamide therapy urine electrophoresis (c)

the portal tracts In the gut there was a diffuse infiltration of the lamina propria nodular and focal infiltrations in the submucosa serosa and muscular layers. The esophagus was also infiltrated. In the kidneys there was interstitial infiltration with formation of nodular areas In the skin there was a widespread vascular infiltration without necrosis The bone marrow showed large plasmocytoid infiltration (fig 2b) The adrenals, the pericardium and the heart were also infiltrated by the same type of cells The pathological diagnosis was malignant lymphoma lymphocytic type poorly differentiated with plasmocytoid differentiation

Case 2 J R M., a 56-year-old male who 8 months before entry presented anorexia weight loss and a dull pain over the left hypochondrium Lymph nodes were felt in both axillae The liver was 3 cm and the spleen 13 cm below the costal margin. At lapartomy several lymph node enlargements were seen in the celiac area and retroperitoneal space Splenectomy lymph node liver kidney and bone marrow biopsies were performed After the operation cyclophosphamide 100 mg daily was instituted and the patient improved. 8 months later he is doing well

---

Fig 2 *a* Lymph node from case 1 widespread proliferation of pleomorphic cells, mostly lymphocytes. HE 375 *b* Bone marrow from case 1, diffuse infiltration by plasmocytic cells He 600 *c* Spleen from case 2 marked plasmocytoid differentiation at the right side of the picture HE 600

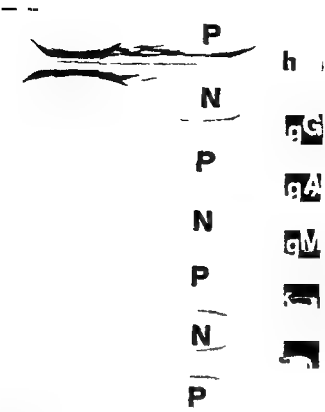


Fig 4 Immunoelectrophoresis in agarose gel of case 2 showing a polyclonal increase of immunoglobulins specially IgG and IgM h = Antihuman antiserum IgG = anti IgG antiserum IgA = anti IgA antiserum k = anti $\varkappa$ antiserum l = anti λ antiserum P = patient s serum N = normal serum

Laboratory results Hematocrit 35%, hemoglobin 11 4 g/100 ml white cells 11 000 with 1% eosinophils 9% band forms 67% neutrophils 21% lymphocytes and 2% monocytes Blood urea nitrogen 88 mg/100 ml creatinin 1 6 mg/100 ml uric acid 12 mg/100 ml total proteins 9 2 g/100 ml with 20 23% albumin 2 35% α_1 8 92% α_2 5.35% β and 63 69% heterogeneous γ globulin IgG was 9 000 mg 100 ml (normal 900–1 800) IgM 900 mg/100 ml (normal 65–150) and IgA 195 mg 100 ml (normal 150–300) Immunoelectrophoresis showed a polyclonal increase of IgG and Igm and no M component could be seen in serum (Fig 4) In the urine proteins were 1 800 mg/24 h with 14 41% albumin 6.51% α 18.5% β and 60 46% γ globulins (fig 3c) By urine immunoelectrophoresis no free light or heavy chains were detected Bone marrow aspiration showed 22% plasmocytic cells with normal erythroid and myeloid series Immunoelectrophoresis of spleen and lymph node homogenates showed high concentrations of IgG and IgM 2 months after splenectomy the immunoglobulins decreased to half of the previous values total proteins 7 4 g 100 ml with 36 72% albumin 2 16% α_1 8 64% α_2 10 80% β and 40 32% γ-globulins

(fig. 3b). 6 months later on continuous cyclophosphamide therapy the serum electrophoresis showed a less marked hypergammaglobulinemia (total proteins 8.3 g/100 ml, 49.3% albumin, 1.2% α_1, 7.7% α_2, 11.6% β and 29.8% γ globulins).

Pathological study. In the lymph nodes there was a widespread proliferation of lymphoid elements and some plasmocytoid cells. The peripheral sinuses were almost full of these cells, and most of the medullary sinuses were also involved. There were heavy iron deposits in the preserved areas. In the spleen the folliculi were big and formed by round monomorphic cells with the chromatin forming clumps. The cytoplasm was scanty. Sinusoids were invaded by the same cells with areas of plasmocytoid differentiation (fig. 2c). The liver showed widespread infiltrations of portal tracts by round lymphoid cells. In the kidneys there were areas of lymphoid and plasmocytoid infiltration in the interstitial space. In the bone marrow myeloid and erythroid series were well preserved and there was only a mild plasmocytic reaction. The pathological diagnosis was malignant lymphoma, lymphocytic type well differentiated with plasmocytoid differentiation.

Discussion

Classification of non-Hodgkin lymphomas is under active revision [1, 5, 10, 14, 18]. Among B cell lymphomas are included all diseases characterized by an abnormal production of immunoglobulins, and this is a well known feature of lymphoplasmocytic proliferative disorders, although not always immunoglobulins are secreted [18]. They usually belong to one type of Ig due to the monoclonal character of the neoplasm [7, 8, 18].

Our 2 patients had clinical and pathological data which merit consideration. The finding of an important polyclonal gammopathy is unusual in neoplastic diseases other than hepatoma [2]. In our second case the diminution of the Ig after splenectomy and cyclophosphamide therapy and the high concentrations of IgG and IgM in spleen and lymph node homogenates seem to confirm the relation between the neoplasm and the Ig secretion. The plasmocytic proliferation in the bone marrow could be compatible with multiple myeloma, but the widespread infiltration of lymph nodes, liver, spleen and kidneys without osseous lesions and the type of dysproteinemia do not support this diagnosis.

Recently, FRIZZERA *et al.* [4] described a group of patients of what they call angioimmunoblastic lymphadenopathy with dysproteinemia, and this observation has been confirmed by several other investigators [6, 11, 12, 14, 17]. The histological picture suggests a host versus-graft type of reaction. In our patients the possibility of an immune reaction of humoral

type against the tumor may not be dismissed, although the histological picture is different of that described by FRIZZERA *et al* [4] and [H RAPPAPORT, personal commun]

KIM *et al* [7], in their revision of lymphoproliferative disorders with monoclonal gammopathies, found 6 cases with a moderate polyclonal increase of IgM and pronounced plasmocytoid features which may well be a less severe form at the same type of clinicopathological picture FLANDRIN *et al* [3] reported 7 patients of what they call plasmocytic sarcomatosis in whom there was also a polyclonal hyperimmunoglobulinemia, although their clinical records were different from those of our patients In Hodgkin's disease a splenic synthetsis of IgG stimulated by tumor-related antigens has been demonstrated [9], but this diagnosis cannot be maintained in our 2 cases Neither of them belong to the double macroglobulinemia-myeloma gammopathy recently reviewed [13] Heavy chain diseases can be excluded because the serum immunoelectrophoresis and urine in case 2 and of the serum with monospecific antiserum, did not show free heavy chains

The widespread proliferative disease in our 2 cases seems to correspond to a malignant neoplastic disorder with polyclonal gammopathy Although specific B cell markers were not determined it is suggested that the tumor may arissey in B cells As recently suggested by SALMON and SELIGMAN [16] this may be a B_2 cell type of neoplasm Only reports of further cases of such a type of disease will confirm this

References

1 BENNET, M H , FARRER BROWN G , HENRY, K , and LELIFE A M Classification of non Hodgkin s lymphomas Lancet *ii* 406 (1974)

2 FENOGLIO, C., PERENCY, A , ISOBE, T , and OSSERMAN E. F Hepatoma associated with marked plasmacytosis and polyclonal hypergammaglobulinemia Am J Med *55* 111 (1973)

3 FLANDRIN G , DANIEL, M T , EL YAFI G et CHELLOUL. Sarcomatoses ganglionnaires diffuses à différentiation plasmocytaire avec anémie hémolytique autoimmune Actual hémat *6* 25 (1972)

4 FRIZZERA G , MORAN E. M., and RAPPAPORT, H Angio-immunoblastic lymphadenopathy with dysproteinaemia Lancet *i* 1070 (1974)

5 GERARD-MARCHANT, R , HAULEN I , LENNERT, K., and RILKE F : Classification of non Hodgkin s lymphomas. Lancet *ii* 407 (1974)

6 HORNE, C H W , FRASER, R A., and PETRIE, J C. Angio-immunoblastic lymphadenopathy with dysproteinaemia Lancet *ii* 291 (1974)

7 Kim A, Heller P, and Rappaport, H Monoclonal gammapathies associated with lymphoproliferative disorders A morphologic study Am. J clin Path 59 282 (1973)
8 Krauss S and Sokal, O E. Paraproteinemia in the lymphomas Am J Med 40 400 (1966)
9 Lowquire, R L. McMillan R., and Yelenosky R., *In vitro* splenic IgG synthesis in Hodgkin s disease New Engl J Med 289 763 (1973)
10 Lukes R J and Tindle, B H Workshop on classification of non Hodgkin s lymphomas (University of Chicago 1973)
11 Lukes, R J and Tindle, B H Immunoblastic lymphadenopathy a hyperimmune entity resembling Hodgkin s disease New Engl J Med 292 1 (1975)
12 Nomanbhoy Y T and Prager R. R Angioimmunoblastic lymphadenopathy with dysproteinemia Lancet *ii* 409 (1974)
13 Prudzanski W, Underdown B and Silver E. H *et al* Macroglobulinemia myeloma double gammapathy A study of four cases and a review of the literature Am J Med 57 259 (1974)
14 Rappaport H in Atlas of tumor pathology sect 3 fasc 8 (Washington 1966)
15 Rappaport H and Moran E. M Angio-immunoblastic (immunoblastic) lymphadenopathy New Engl J Med 292 42 (1975)
16 Salmon S L. and Seligmann M B-Cell neoplasia in man Lancet *ii* 1230 (1974)
17 Schultz, D R and Yunis A A Immunoblastic lymphadenopathy with mixed cryoglobulinemia New Engl J Med 292 8 (1975)
18 Stein H Lennert K., and Parwaresch M R Malignant lymphomas of B-cell type Lancet *ii* 855 (1972)

Dr J Guardia Avda de la Victoria 64 *Barcelona 17* (Spain)

Acta haemat. 55: 354–357 (1976)

Splenunculectomy in Recurrent Thrombocytopenia[1]

JOEL A. SPERO, JESSICA H. LEWIS, UTE HASIBA and L. W. GUMERMAN

Departments of Medicine and Radiology (Nuclear Medicine Section), University of Pittsburgh, and Presbyterian University Hospital and Central Blood Bank of Pittsburgh, Pittsburgh, Pa.

Key Words. Accessory spleen · ITP · Splenectomy · Splenunculectomy · Thrombocytopenia

Abstract. A 13-year-old boy developed thrombocytopenic purpura in 1953 which improved during 4 weeks of cortisone therapy. Following 13 years of intermittent symptoms the platelet count was found to be 8,000/µl. After splenectomy the patient was asymptomatic for 8 years, but had a recurrence of symptoms and thrombocytopenia in 1974. An initial spleen scan with ^{99}Tcm sulfur colloid was negative, but when repeated with a gamma camera and shielded liver, a splenunculus was demonstrated. After splenunculectomy a rapid remission occurred, and the patient has been well for 13 months.

The search for and removal of an accessory spleen (lienculus, lienunculus, splenculus, splenulus or splenunculus) in a patient with recurrent thrombocytopenia following *splenectomy* seems to be a forgotten mode of treatment for this recalcitrant disease. The last reports and/or reviews [2, 8, 9] were published in the 1950s. Current literature emphasizes the use of immunosuppressive agents or corticosteroids [3, 6]. This case is presented as a reminder that it is still worthwhile hunting for a splenunculus because its excision may induce a rapid remission.

Case History

The patient is a 35-year-old male who, in July 1953 at age 13, was diagnosed as having acute idiopathic thrombocytopenic purpura (ITP) which responded to steroid

[1] Supported in part by research grant HL-02254 from the National Institutes of Health.

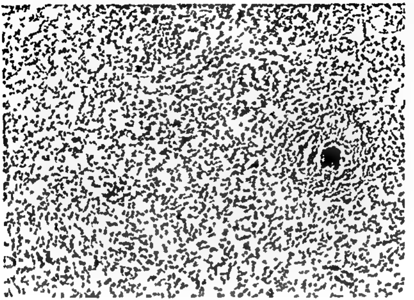


Fig. 1. Microscopic section of splenunculus ... 75

therapy. In the spring of 1966 he was contacted and asked to come to this laboratory as part of a study of individuals who had had ITP [10]. A history of intermittent episodes of spontaneous petechiae, easy bruising, gum bleeding and epistaxis during the past 13 years was obtained. No treatment had been instituted. Physical examination showed ecchymotic areas on both arms and one thigh. Spleen was not palpable. Platelet count was 8 000/ l and bone marrow aspiration and biopsy revealed a normal number of megakaryocytes. LE preparation was negative.

He was treated with prednisone, 100 mg/day. Because he was unable to maintain safe platelet levels when steroids were tapered, a splenectomy was performed in December 1966. The spleen weighed 130 g and histology was normal. An accessory spleen weighing 0.5 g was also excised at surgery. Within days his platelet count rose to greater than a million. Steroids were stopped and he was followed for 2 years during which time he maintained platelet counts greater than 350 000/ l. He remained asymptomatic for the ensuing 5 years, and during a hospitalization for a herniorrhaphy in 1973, his platelet count was reported as normal. In mid April 1974, more than 7 years post splenectomy, he noted a recurrence of easy bruisability but did not seek medical advice until late in May when his platelet count was found to be 25 000/ l. Treatment with prednisone resulted in a temporary increase in platelet count, but by early July 1974 his count again fell markedly with tapering and of the medication. In mid July a conventional rectilinear scan of the upper abdomen using 99Tcm sulfur colloid showed no significant concentration of radioactivity outside of the liver. Because the possibility of an accessory spleen was strongly suspected, the

study was repeated 2 weeks later utilizing a gamma-camera. Images were obtained of the left upper quadrant with lead placed over the liver, thereby emphasizing the recording of extrahepatic radioactivity. With this technique a significant amount of the radiopharmaceutical was demonstrated in the splenic bed, and it was felt to represent an accessory spleen of approximately 20 g. On August 6, 1974, a laparotomy was performed and an accessory spleen weighing 12.8 g was found (fig. 1). Prolonged search revealed no other macroscopic splenic tissue. Following surgery his platelet count rose to a peak of 455,000/μl and was 290,000 13 months after operation. The patient was clinically well at the time of his most recent platelet count.

Discussion

In the early 1950s several reports appeared in the literature discussing the importance of accessory spleens in recurrent ITP. Since that time it appears that interest in pursuing a splenunculus in this setting has waned. Considering the marked improvement in diagnostic procedures in the last 20 years, especially in the area of nuclear medicine, the tendency to move directly to drug therapy without exhaustive search for residual splenic tissue may well be doing a disservice to a small percentage of patients with recurrent ITP.

The presence of accessory spleens in necropsy series varies from 10 [4] to 31% [1], however, their occurrence following splenectomy is not as well documented. It has been shown that more than 10% of those patients with one accessory spleen have been found to have additional splenunculi [1, 4, 5], the implication being that a patient who has an accessory spleen at initial surgery has at least a 10% chance of having additional aberrant splenic tissue. Howell-Jolly bodies were not seen in this patient's peripheral blood smears, and this was taken as an additional indicator of the presence of functioning splenic tissue.

These two factors, i.e. the previous finding of a splenunculus and the lack of Howell-Jolly bodies, led to the heightened index of suspicion that prompted continued searching following the initial negative scan and the subsequent radiologic demonstration of the existence of an accessory spleen. The prior intention in this patient had been to proceed with a heat-treated, ^{51}Cr-labeled red blood cell study [7] if the repeat ^{99}Tc^{m} spleen scan using a long exposure gamma camera and liver shielding had been negative. This procedure is based upon the observation that the splenic uptake of red blood cells is said to be significantly greater than that of the liver. If all studies had been negative, in all likelihood drug

study was repeated 2 weeks later utilizing a gamma-camera. Images were obtained of the left upper quadrant with lead placed over the liver, thereby emphasizing the recording of extrahepatic radioactivity. With this technique a significant amount of the radiopharmaceutical was demonstrated in the splenic bed and it was felt to represent an accessory spleen of approximately 20 g. On August 6, 1974, a laparotomy was performed and an accessory spleen weighing 12.8 g was found (fig. 1). Prolonged search revealed no other macroscopic splenic tissue. Following surgery his platelet count rose to a peak of 455 000/μl and was 250 000 13 months after operation. The patient was clinically well at the time of his most recent platelet count.

Discussion

In the early 1950s several reports appeared in the literature discussing the importance of accessory spleens in recurrent ITP. Since that time it appears that interest in pursuing a splenunculus in this setting has waned. Considering the marked improvement in diagnostic procedures in the last 20 years, especially in the area of nuclear medicine, the tendency to move directly to drug therapy without exhaustive search for residual splenic tissue may well be doing a disservice to a small percentage of patients with recurrent ITP.

The presence of accessory spleens in necropsy series varies from 10 [4] to 31% [1], however, their occurrence following splenectomy is not as well documented. It has been shown that more than 10% of those patients with one accessory spleen have been found to have additional splenunculi [1, 4, 5], the implication being that a patient who has an accessory spleen at initial surgery has at least a 10% chance of having additional aberrant splenic tissue. Howell-Jolly bodies were not seen in this patient's peripheral blood smears, and this was taken as an additional indicator of the presence of functioning splenic tissue.

These two factors, i.e. the previous finding of a splenunculus and the lack of Howell-Jolly bodies, led to the heightened index of suspicion that prompted continued searching following the initial negative scan and the subsequent radiologic demonstration of the existence of an accessory spleen. The prior intention in this patient had been to proceed with a heat-treated, ^{51}Cr-labeled red blood cell study [7] if the repeat ^{99}Tc^{m} spleen scan using a long exposure gamma camera and liver shielding had been negative. This procedure is based upon the observation that the splenic uptake of red blood cells is said to be significantly greater than that of the liver. If all studies had been negative, in all likelihood drug

therapy would have been continued Random exploratory laparotomies in the absence of evidence for splenic activity are not justifiable

A vigorous search for an accessory spleen in all patients with recurrent thrombocytopenia following splenectomy induced remission, especially those in whom the index of suspicion is raised, may avoid the potential hazard and expense of prolonged chemotherapy

References

1 Curtis G M and Movitz D The surgical significance of the accessory spleen Ann Surg *123* 276–298 (1946)

2 Evans T S Spinner S Piccolo P., *et al* Recurrent hypersplenism due to accessory spleen Acta haemat *10* 350–359 (1953)

3 Finch S C Castro O, Cooper M., *et al* Immunosuppressive therapy of chronic idiopathic thrombocytopenic purpura Am J Med *56* 4–12 (1974)

4 Halpert B and Györkey F Lesions observed in accessory spleens of 311 patients Am J clin Path *32* 165–168 (1959)

5 Halpert B and Alden Z A Accessory spleens in or at the tail of the pancreas Archs Path *77* 652–654 (1964)

6 Jui R M Firozvi T., and Spurling C L Chronic idiopathic thrombocytopenic purpura Archs intern Med *132* 390 393 (1973)

7 Johnson P M Wood J H., and Moorno S I Splenic scintillation scanning. Am J Roentg III 757 767 (1961)

8 Loeb V Seaman W B., and Moore, C V The use of thorium dioxide sol (thorotrast) in the roentgenologic demonstration of accessory spleens. Blood 7 904 914 (1952)

9 Rosenthal N Vogel P Lee S *et al* The role of accessory spleens in post splenectomy recurrent purpura hemorrhagica J Mt Sinai Hosp *17* 1008 1020 (1951)

10 Szeto I L F Bayer W L Kuhl, C G., *et al* Idiopathic thrombocytopenic purpura in Ambrus Hematologic reviews vol 1 pp 51–67 (Dekker New York 1968)

Joel A Spero MD Central Blood Bank of Pittsburgh, 812 Fifth Avenue *Pittsburgh PA 15219* (USA)

Acta haemat. 55 358–362 (1976)

Multiple Myeloma with Terminal Erythroleukemia

DINA MEYTES, URI SELIGSOHN and BRACHA RAMOT

Institute of Hematology, Chaim Sheba Medical Center, Tel Hashomer, and Sackler School of Medicine, Tel-Aviv University, Tel-Aviv

Key Words Erythroleukemia · Melphalan · Myeloma · Preleukemia

Abstract. A patient with multiple myeloma treated by melphalan terminated in erythroleukemia. The evolution of the leukemia was preceded by a protracted preleukemic phase, as evidenced by progressive pancytopenia. The possible implications of an early diagnosis of preleukemia in myeloma are discussed.

A number of case reports of acute myeloid or myelomonocytic leukemia as the terminal event in multiple myeloma have been published [6]. Recently, sideroblastic anemia was described as a forerunner of this leukemia [3]. The following report presents the evolution of erythroleukemia in a patient with multiple myeloma.

Case Report

This 72 year old Jewish female was diagnosed in 1965 as suffering from multiple myeloma. Except for cholelithiasis and recurrent urinary tract infections, the past history was uneventful. On admission she had generalized osteoporosis, an asymptomatic compression fracture of L_1 vertebra and osteolytic lesions of the skull. Anemia of 9 g% hemoglobin and hyperglobulinemia with 4.5 g% globulins were present. The rest of the blood count, blood chemistry and urinalysis were normal. Serum electrophoresis demonstrated a monoclonal gammopathy. This was identified as IgA/λ. The bone marrow aspirate was hypercellular and contained 50% mature plasma cells, normal myeloid series and normoblastic erythropoiesis. Since this relatively old lady was asymptomatic, no specific treatment was instituted.

In November 1969, following a nailing for a hip fracture and gram negative septicemia, an episode of hypercalcemic coma developed. A prompt response was

Table I The patient's course: serial blood counts, immunoglobulin levels and melphalan therapy

Period	Peripheral blood					Immunoglobulins, mg%		
	hemoglobin g%	leucocytes/mm³	platelets/mm³	blasts %	normoblasts per 100 WBC	IgA	IgG	IgM
1965–1969	10.6–11.0	4,000–5,700	180,000–200,000	0	0			
Melphalan								
Apr 70–Jan 73[1]	9.7–13.1	3,500–6,300	110,000–230,000	0	0	4,350	180	47
Jan 73–Sep 73[2]	11.7–12.0	2,400–5,300	102,000–211,000	0	0			
Oct 73–Feb 74[1]	10.5–8.0	2,100–6,300	71,000–120,000	0	0	300	680	95
Apr 74–Dec 74[3]	8.0–5.0	2,100–3,500	112,000–12,000	1–7	25–60			

[1] Cyclic melphalan therapy without interruption.
[2] Melphalan repeatedly discontinued every 2–3 months.
[3] Melphalan completely withdrawn.

achieved by buffer phosphate infusion and prednisone. At that time immunoglobulin levels were 4,350 mg% IgA, 180 mg% IgG and 47 mg% IgM. Subsequently cyclic therapy with melphalan (2–4 mg daily for 1 week with 2 weeks off therapy) and intermittent prednisone was instituted. On this regimen the patient remained asymptomatic during the following 4 years. The hemoglobin rose to 13 g%, and the IgA of the monoclonal type decreased gradually to 300 mg%, with a concomitant rise in IgG to 680 mg% and IgM to 95 mg% (table I).

Starting from January 1973, melphalan had to be repeatedly discontinued because of leucopenia. Since October the hemoglobin level started to drop progressively, and in April 1974 a persistent pancytopenia developed. Numerous normoblasts (from 25 to 50/100 WBC), poikilocytes, schistocytes, basophilic stippling and occasional myeloblasts were visible on the blood smear. Most of the normoblasts were ringed sideroblasts and 30% were strongly PAS positive. The bone marrow aspirate was hypercellular with only 5% mature plasma cells. The erythroid series was markedly hyperplastic with a shift to the left. Numerous double nucleated normoblasts with prominent siderotic granules and ringed sideroblasts were present. In the myeloid series, except for a shift to the left with up to 1% blasts, no abnormality was observed. The serum iron was 166 mg%, and IBC 133 mg%, and the LAP score 244/100 WBC.

In view of the pancytopenia melphalan therapy was discontinued and the patient remained asymptomatic on 30 mg prednisone daily; however, the blood count dropped further. The serial counts are presented in table I. Cytogenetic examination of the peripheral blood, performed in December 1974, demonstrated 2 aberrant metaphases out of 10 scorable cells: 46,xx,G+F and 47,xx+C, respectively. In the repeated bone marrow the erythropoiesis was even more pathological, with triple nu-

Acta haemat. 55: 358–362 (1976)

Multiple Myeloma with Terminal Erythroleukemia

DINA MEYTES, URI SELIGSOHN and BRACHA RAMOT

Institute of Hematology, Chaim Sheba Medical Center, Tel Hashomer, and Sackler School of Medicine, Tel Aviv University, Tel Aviv

Key Words. Erythroleukemia · Melphalan · Myeloma · Preleukemia

Abstract. A patient with multiple myeloma treated by melphalan terminated in erythroleukemia. The evolution of the leukemia was preceded by a protracted preleukemic phase, as evidenced by progressive pancytopenia. The possible implications of an early diagnosis of preleukemia in myeloma are discussed.

A number of case reports of acute myeloid or myelomonocytic leukemia as the terminal event in multiple myeloma have been published [6]. Recently, sideroblastic anemia was described as a forerunner of this leukemia [3]. The following report presents the evolution of erythroleukemia in a patient with multiple myeloma.

Case Report

This 72-year-old Jewish female was diagnosed in 1965 as suffering from multiple myeloma. Except for cholelithiasis and recurrent urinary tract infections, the past history was uneventful. On admission she had generalized osteoporosis, an asymptomatic compression fracture of L_1 vertebra and osteolytic lesions of the skull. Anemia of 9 g% hemoglobin and hyperglobulinemia with 4.5 g% globulins were present. The rest of the blood count, blood chemistry and urinalysis were normal. Serum electrophoresis demonstrated a monoclonal gammopathy. This was identified as IgA/κ. The bone marrow aspirate was hypercellular and contained 50% mature plasma cells, normal myeloid series and normoblastic erythropoiesis. Since this relatively old lady was asymptomatic, no specific treatment was instituted.

In November 1969, following a nailing for a hip fracture and gram-negative septicemia, an episode of hypercalcemic coma developed. A prompt response was

Table I The patient's course: serial blood counts, immunoglobulin levels and melphalan therapy

Period	Peripheral blood					Immunoglobulins mg%		
	hemoglobin g%	leucocytes/mm³	platelets/mm³	blasts %	normoblasts per 100 WBC	IgA	IgG	IgM
1965–1969	10.6–11.0	4,000–5,700	180,000–200,000	0	0			
Melphalan								
Apr 70–Jan 73[1]	9.7–13.1	3,500–6,300	110,000–230,000	0	0	4,350	180	47
Jan 73–Sep 73[2]	11.7–12.0	2,400–5,300	102,000–211,000	0	0			
Oct 73–Feb 74[2]	10.5–8.0	2,100–6,300	71,000–120,000	0	0	300	680	95
Apr 74–Dec 74[3]	8.0–5.0	2,100–3,500	112,000–12,000	1–7	25–60			

[1] Cyclic melphalan therapy without interruption
[2] Melphalan repeatedly discontinued every 2–3 months
[3] Melphalan completely withdrawn

achieved by buffer phosphate infusion and prednisone. At that time immunoglobulin levels were 4,350 mg% IgA, 180 mg% IgG and 47 mg% IgM. Subsequently cyclic therapy with melphalan (2–4 mg daily for 1 week, with 2 weeks off therapy) and intermittent prednisone was instituted. On this regimen the patient remained asymptomatic during the following 4 years. The hemoglobin rose to 13 g%, and the IgA of the monoclonal type decreased gradually to 300 mg%, with a concomitant rise in IgG to 680 mg% and IgM to 95 mg% (table I).

Starting from January 1973, melphalan had to be repeatedly discontinued because of leucopenia. Since October the hemoglobin level started to drop progressively, and in April 1974 a persistent pancytopenia developed. Numerous normoblasts (from 25 to 50/100 WBC), poikilocytes, schistocytes, basophilic stippling and occasional myeloblasts were visible on the blood smear. Most of the normoblasts were ringed sideroblasts and 30% were strongly PAS positive. The bone marrow aspirate was hypercellular with only 5% mature plasma cells. The erythroid series was markedly hyperplastic with a shift to the left. Numerous double-nucleated normoblasts with prominent siderotic granules and ringed sideroblasts were present. In the myeloid series, except for a shift to the left with up to 1% blasts, no abnormality was observed. The serum iron was 166 mg% and IBC 133 mg% and the LAP score 244+/100 WBC.

In view of the pancytopenia melphalan therapy was discontinued, and the patient remained asymptomatic on 30 mg prednisone daily; however, the blood count dropped further. The serial counts are presented in table I. Cytogenetic examination of the peripheral blood, performed in December 1974, demonstrated 2 aberrant metaphases out of 10 scorable cells: 46,XX,G+,E– and 47,XX,+C, respectively. In the repeated bone marrow the erythropoiesis was even more pathological, with triple nu

Acta haemat 55 355–362 (1976)

Multiple Myeloma with Terminal Erythroleukemia

DINA MEYTES, URI SELIGSOHN and BRACHA RAMOT

Institute of Hematology, Chaim Sheba Medical Center, Tel Hashomer, and Sackler School of Medicine, Tel Aviv University, Tel Aviv

Key Words Erythroleukemia Melphalan Myeloma Preleukemia

Abstract A patient with multiple myeloma treated by melphalan terminated in erythroleukemia The evolution of the leukemia was preceded by a protracted preleukemic phase, as evidenced by progressive pancytopenia The possible implications of an early diagnosis of preleukemia in myeloma are discussed

A number of case reports of acute myeloid or myelomonocytic leukemia as the terminal event in multiple myeloma have been published [6] Recently, sideroblastic anemia was described as a forerunner of this leukemia [3] The following report presents the evolution of erythroleukemia in a patient with multiple myeloma

Case Report

This 72 year old Jewish female was diagnozed in 1965 as suffering from multiple myeloma Except for cholelithiasis and recurrent urinary tract infections the past history was uneventful On admission she had generalized osteoporosis, an asymptomatic compression fracture of L_1 vertebra and osteolytic lesions of the skull Anemia of 9 g% hemoglobin and hyperglobulinemia with 4.5 g% globulins were present The rest of the blood count, blood chemistry and urinalysis were normal Serum electrophoresis demonstrated a monoclonal gammopathy This was identified as IgA/λ The bone marrow aspirate was hypercellular and contained 50% mature plasma cells, normal myeloid series and normoblastic erythropoiesis Since this relatively old lady was asymptomatic, no specific treatment was instituted

In November 1969, following a nailing for a hip fracture and gram negative septicemia an episode of hypercalcemic coma developed A prompt response was

Table I The patient's course serial blood counts, immunoglobulin levels and melphalan therapy

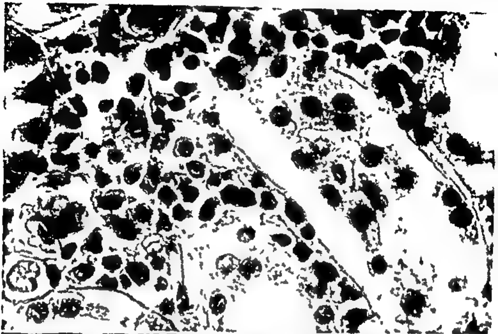
Period	Peripheral blood					Immunoglobulins, mg%		
	hemoglobin g%	leucocytes/mm³	platelets/mm³	blasts %	normoblasts per 100 WBC	IgA	IgG	IgM
1965 1969	10.6–11.0	4,000–5,700	180,000–200,000	0	0			
Melphalan								
Apr 70 Jan 73[1]	9.7–13.1	3,500–6,300	110,000–230,000	0	0	4,350	180	47
Jan 73 Sep 73[2]	11.7–12.0	2,400–5,300	102,000–211,000	0	0			
Oct 73 Feb 74[2]	10.5–8.0	2,100 6,300	71,000–120,000	0	0	300	680	95
Apr 74 Dec 74[3]	8.0–5.0	2,100–3,500	112,000–12,000	1 7	25–60			

[1] Cyclic melphalan therapy without interruption
[2] Melphalan repeatedly discontinued every 2 3 months
[3] *Melphalan completely withdrawn*

achieved by buffer phosphate infusion and prednisone At that time immunoglobulin levels were 4,350 mg% IgA 180 mg% IgG and 47 mg% IgM Subsequently, cy*clic therapy with melphalan (2–4 mg daily for 1 week with 2 weeks off therapy)* and intermittent prednisone was instituted On this regimen the patient remained asymptomatic during the following 4 years. The hemoglobin rose to 13 g%, and the IgA of the monoclonal type decreased gradually to 300 mg% with a concomitant rise in IgG to 680 mg% and IgM to 95 mg% (table I)

Starting from January 1973 melphalan had to be repeatedly discontinued because of leucopenia Since October *the hemoglobin level started to drop progressively* and in April 1974 a persistent pancytopenia developed. Numerous normoblasts (from 25 to 50/100 WBC) poikilocytes, schistocytes, basophilic *stippling* and occasional myeloblasts were visible on the blood smear Most of the normoblasts were ringed sideroblasts and 30% were strongly PAS positive The bone marrow aspirate was hypercellular with only 5% mature plasma cells The erythroid series was mark*edly hyperplastic with a shift to the left Numerous* double nucleated normoblasts with prominent siderotic granules and ringed sideroblasts were present In the myeloid series, except for a shift to the left with up to 1% blasts no abnormality was observed The serum iron was 166 mg% and IBC 133 mg% and the LAP score *244/100 WBC*

In view of the pancytopenia melphalan therapy was discontinued, and the patient remained asymptomatic on 30 mg prednisone daily, however the blood count dropped further The serial counts are presented in table I Cytogenetic examination of the peripheral blood performed in December 1974 demonstrated 2 aberrant metaphases out of 10 scorable cells 46,xx-G+1 and 47,xx+C respectively In the repeated bone marrow the erythropoiesis was even more pathological with triple nu

1


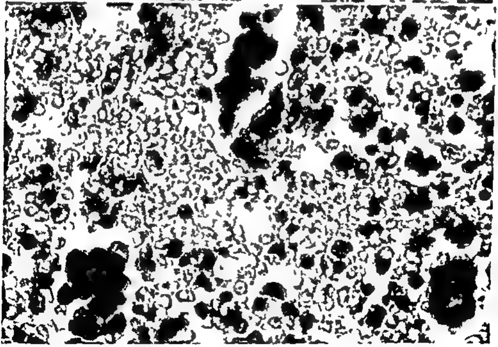
2


Fig 1 Erythroleukemic infiltrate in kidney HE × 630

Fig 2 Clusters of abnormal erythropoietic cells in spleen HE × 1,000

cleated normoblasts and megaloblasts. Also the megakaryocytes showed asynchronic maturation.

The patient died of a massive bronchopneumonia by the end of December 1974. At autopsy erythroleukemic infiltrates were found in the bone marrow, spleen, liver, kidney and occasional lymph nodes (fig. 1, 2).

Discussion

Erythroleukemia, a rare form of acute leukemia, is more frequent following potentially leukemic conditions. Thus, 3 of 6 patients with leukemia post polycythemia vera [5], 3 of 7 patients with leukemia following macroglobulinemia [1, 6] and 4 of 74 patients with leukemia post Hodgkin's disease [7] terminated in erythroleukemia. However, in a recent review of leukemia post myeloma only in 1, of all 46 cases culled from the literature, erythroleukemia evolved [6].

This patient demonstrated a number of additional aspects: during a period of 4 years she had a documented multiple myeloma, including bone destruction. However, since the policy of this department is not to treat subjectively asymptomatic patients, she was followed for 4 years without any specific therapy. Due to a fracture followed by septicemia with hypercalcemic coma the patient was put on melphalan, using a relatively conservative schedule. She responded remarkably, the pathological protein dropped significantly, the normal immunoglobulins and the hemoglobin rose and the patient resumed normal life. Therefore, at least in this case, the delay in instituting therapy did not change her responsiveness to melphalan. This appears to be quite a consistent observation in our material [4]. The last stage of the disease was characterized by difficulties in pursuing melphalan therapy because of repeated pancytopenia. Finally, erythroleukemia evolved. Retrospectively, it is clear that the cytopenia was related to the preleukemic phase, and was not due to myelosuppression by the drug.

A preleukemic phase, consisting of pancytopenia and sideroblastic marrow, has recently been described preceding myelomonocytic leukemia [8], and the acute leukemia post multiple myeloma [3], as well as post macroglobulinemia [1], polycythemia vera [5] and Hodgkin's disease [9]. Therefore, preleukemia should be borne in mind when considering the various causes of cytopenias in patients who receive myelosuppressive therapy for potentially leukemic conditions. In view of the strong implication of melphalan in the evolution of acute leukemia in multiple myeloma

[2], the demonstration of preleukemia should call for a complete withdrawal of this drug

References

1 Cardamone, J M, Kimmerle, R I, and Marshall, E Y Development of acute erythroleukemia in B cell immunoproliferative disorders after prolonged therapy with alkylating drugs Am J Med 57 836 842 (1974)
2 Holland, J F Epidemic acute leukemia New Engl J Med 283 1165 (1970)
3 Khaleeli M, Keane, W M and Lee, G R Sideroblastic anemia in multiple myeloma – a preleukemic change Blood 41 17–25 (1973)
4 Levo, Y, Many, A, and Ramot, B Personal commun
5 Meytes, D, Katz, D, and Ramot, B Preleukemia and leukemia in polycythemia vera Blood (in press)
6 Rosner, F and Grünwald, H Multiple myeloma terminating in acute leukemia report of 12 cases and review of the literature Am J Med 57 927–939 (1974)
7 Rosner, F and Grünwald, H Hodgkin s disease and acute leukemia report of 8 cases and review of the literature Am J Med 59 339–353 (1975)
8 Saarni, M I and Linman, J W Preleukemia the hematologic syndrome preceding acute leukemia Am J Med 55 38–48 (1973)
9 Steinberg, M H, Geary, C G., and Crosby, W H Acute granulocytic leukemia complicating Hodgkin's disease Archs intern Med 125 496–498 (1970)

B Ramot, MD, Professor of Medicine, Head, Institute of Hematology, Chaim Sheba Medical Center, *Tel Hashomer* (Israel)

Index rerum ad Vol. 55

Bearbeitet von G. Botush, Basel
(B) = Book Reviews – Buchbesprechungen – Livres nouveaux

Index autorum ad Vol. 55

(B) = Book Reviews – Buchbesprechungen – Livres nouveaux

Table V. Patient's lymphocyte stimulation in culture using penicillin, ampicillin and cephalothin as antigen performed on 2 different days (mean of 2 tests)

	16.8.70 cpm	18.9.70 cpm
Lymphocytes alone	513	472
Lymphocytes + penicillin G	971	728
Lymphocytes + ampicillin	1,119	884
Lymphocytes + cephalothin	1,096	1,216
Lymphocytes + PPD (10 μ)	1,582	1,390
Lymphocytes + phytohemagglutinin	63,620	54,620

way. Moreover (table IV), no clear-cut difference could be seen between the fractions from the patient's serum and those from normal serum. The serum proteins most readily absorbed by this apparently nonimmunologic mechanism were IgG, IgA, transferrin and β-lipoprotein.

Evidence for sensitization to cephalothin. Skin tests were performed in the patient, 9 days after therapy with cephalothin had been interrupted. A positive skin test was observed after intradermal injection of penicilloyl-polylysine, 250 mmol/ml), the last test was stronger than the others. Lymphocyte culture tests were also performed on two different days, using the same three drugs as antigens (table V). All three tests induced moderately positive lymphocyte stimulation.

Discussion

The reported incidence of positive Coombs test in patients receiving cephalothin has varied between 3.4 and 75% [4, 7, 9, 11]. These reactions were often weak and occurred from one to a few days after starting treatment. They were not associated with hemolysis. In some studies, the dosage and duration of therapy were found to influence the incidence of positive reactions. A large percentage of patients with positive Coombs tests had renal disease with high blood levels of cephalothin secondary to reduced renal excretion. As suggested by GRALNICK *et al.* [4], hypoalbuminemia may also favor the occurrence of positive Coombs tests, the albumin fraction being responsible for over 95% of the serum binding of cephalothin [6].

Previously proposed mechanisms regarding the pathogenesis of posi-

tive Coombs test without hemolysis, occurring in patients receiving cephalothin include (1) a nonimmunologic binding of serum proteins to red cells [4, 7, 11] and (2) an immune reaction involving an antibody cross reacting with cephalothin or penicillin-coated red cells [4, 8, 11] As indicated by SPATH *et al* [11], both mechanisms seem to occur in parallel in these patients Nonimmunological absorption of proteins, especially albumin, to cephalothin-coated red cells could be demonstrated *in vitro*, multiple absorptions of a serum with cephalothin sensitized erythrocytes revealed that these cells absorb IgG and other proteins, including complement (C3) and albumin [11] Our own data confirm this nonimmunologic binding of proteins and revealed that IgA, transferrin and β lipoprotein may also be absorbed from normal sera by cephalothin-coated erythrocytes (table III) In addition SPATH *et al* [11] have demonstrated that an immune mechanism may also cause positive direct antiglobulin tests *in vivo* during cephalothin therapy Eluates from the patient s erythrocytes react with cephalotin and penicillin sensitized erythrocytes This was not a nonspecific absorption of protein because eluates from red cells sensitized with an anti Rh IgG isoantibody would not bind IgG to cephalothin-coated red cells lacking the appropriate antigen In addition, SPATH *et al* [11] showed that the sera of 4 patients having a positive antiglobulin test all contained high titers of IgG cephalothin antibodies, whereas in the other treated patients, IgM antibodies were found in 88% and IgG antibodies in only 25% of cases

Despite the frequent occurrence of a positive direct Coombs test in patients receiving cephalothin only 2 cases of hemolytic anemia associated with cephalothin have been described so far [5] As in our case, erythrocytes from these patients were strongly positive in the direct Coombs test with broad spectrum anti human globulin reagents and with a specific anti IgG antiserum Our patient s erythrocytes were also strongly agglutinated by an anti-complement (C3) reagent Weak reactions were also obtained with anti IgM anti IgA and anti transferrin reagents, suggesting the simultaneous occurrence of nonimmunological binding of serum proteins In our case as in the others a specific IgG cephalothin antibody was demonstrated in eluates from patients erythrocytes However, as mentioned above this specific immune reaction was also observed in patients with positive direct Coombs test without hemolysis and therefore cannot be used as a definitive proof that cephalothin caused the hemolytic anemia Presumably as suggested by WORLLEDGE [12] the quality and quantity of the antibody are also important.

Several feature suggest that our patient was actually sensitized to cephalothin. Skin tests performed after therapy had been interrupted, revealed sensitization to cephalothin, but also to penicillin and ampicillin, two drugs the patient had received previously. In addition lymphocyte stimulation was observed in culture tests *in vitro* using the same three drugs as antigens. The possible role of cephalothin in hemolytic anemia is further suggested by the fact that evidence of hemolysis was noted within 2 weeks after therapy had been initiated, and disappeared quickly when the drug was stopped. The direct anti-globulin test gradually became weaker until it was found negative, 60–70 days later, when the blood level of cephalothin was very low in the blood and the anemia much improved. This patient illustrates the complexity of interpreting immunological tests involving drug interacting with red blood cell membranes.

Acknowledgements. We thank Miss RACHEL BALLARD and Mr. P. MESLAN for valuable technical assistance.

References

1 CRUCHAUD, A. and JUDITZ, E.: An analysis of gastric parietal cell antibodies and thyroid cell antibodies in patients with pernicious anaemia. Clin. exp. Immunol. *3:* 771 (1968).
2 FORBES, C. D., CRAIG, J. A., MITCHELL, R., and MC NICOL, G. P.: Acute intravascular hemolysis associated with cephalexin therapy. Post grad. med. J. *48:* 186 (1972).
3 GIRARD, J. P., POUTON, M. F., and PRESS, P.: Culture of peripheral blood lymphocytes from sarcoidosis. Response to mitogenic factor. Int. Archs Allergy appl. Immun. *41:* 604 (1971).
4 GRALNICK, H. R., WRIGHT, L. D., and MC GINNIS, M. H.: Coombs' positive reactions associated with sodium cephalothin therapy. J. Am. med. Ass. *199:* 725 (1967).
5 GRALNICK, H. R., MC GINNISS, M. H., ELTON, N., and MC CURDY, P.: Hemolytic anaemia associated with cephalothin. J. Am. med. Ass. *217:* 1193 (1971).
6 LEE, C. C. and ANDERSON, R. C.: Blood and tissue distribution of cephalothin. Antimicrob. Agents Chemother. *2:* 695 (1962).
7 MOLTHAN, L., REIDENBERG, M. M., and EICHMAN, M. F.: Positive direct Coombs tests due to cephalothin. New Engl. J. Med. *277:* 123 (1967).
8 NESMITH, L. W. and DAVIS, J. W.: Hemolytic anemia caused by penicillin. J. Am. med. Ass. *203:* 81 (1968).
9 PERKINS, R. L., MENGEL, C. E., and SASLAW, S.: Direct Coombs' test reactivity after cephalothin or cephaloridine in man and monkey. Proc. Soc. exp. Biol. Med. *129:* 397 (1968).

10 RUBIN H Antibody elution from red blood cells J clin Path *16* 70 (1963)
11 SPATH P, GARRATTY, G., and PETZ, L. Studies on the immune response to penicillin and cephalothin in humans II Immunohematologic reactions to cephalothin administration J Immun *107* 860 (1971)
12 WORLLEDGE, S M Immune drug induced hemolytic anemias Seminars Hematol *10* 327 (1973)

Dr M JEANNET Hôpital cantonal, 64 avenue de la Roseraie *CH 1211 Genève 4* (Switzerland)

Acta haemat *55* 118–122 (1976)

Simultaneous Occurrence of Myeloma and Hodgkin's Disease

Peter V. Sacks, Mehdi Tavassoli and D Ted Eastlund

Division of Hematology and Oncology, Scripps Clinic and Research Foundation, La Jolla, Calif

Key Words *Hodgkin's disease* *Multiple malignancies* *Myeloma* *Paraproteins*

Abstract A patient in whom lymphocyte-depleted Hodgkin's disease and multiple myeloma were diagnosed simultaneously is reported There was no prior history of irradiation or chemotherapy, the significance of which is discussed in relation to previously documented patients with Hodgkin's disease and myeloma

The coexistence of Hodgkin's disease and multiple myeloma has only been reported in three patients [4, 7] In each of these instances, myeloma was diagnosed after either irradiation or chemotherapy had been given for the Hodgkin's disease, raising the possibility that myeloma may heve been induced, or precipitated, by these therapeutic interventions The purpose of this report is to present a fourth patient in whom both Hodgkin's disease and multiple myeloma were diagnosed simultaneously, in the absence of previous irradiation or chemotherapy

Case Report

A 51-year-old fisherman began experiencing recurrent fever up to 40°C in December 1973 associated with intermittent rigors and night sweats A 9 kg weight loss ensued over the following 7 months The fever had a classical Pel Ebstein periodicity

[illegible] hepatosplenomegaly, [illegible] 00 with [illegible] platelet count 250,000/μl, sedimentation rate 107 mm/h, total protein 7 8 g/100 ml, and albumin 3 7 g/100 ml A serum protein electrophoresis showed a monoclonal spike in the gamma

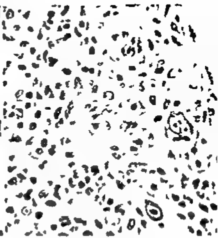


Fig 1 Bone marrow smear stained with Wright-Giemsa, showing abnormal plasma cells with some double nucleated forms containing nucleoli ×930 *Inset* Plasma cell with four nuclei ×705

Fig 2 Section of bone marrow biopsy, stained with hematoxylin-eosin, showing pleomorphic cellular features with abnormal 'reticulum cells', some containing large nucleoli. Note the double nucleated reticulum cell with prominent nucleoli, resembling a Reed-Sternberg cell. ×210

region approximating 1.7 g%. Immunoelectrophoresis revealed a monoclonal IgG-kappa paraprotein with marked attenuation of normal immunoglobulins. Free kappa light chains were detected in the urine in low concentration. A quantitative T- and B-lymphocyte study revealed absolute lymphocytopenia (252/µl) with a relative increase in the numbers of IgG kappa, B-lymphocytes. BUN, calcium, roentgenographic bone density, chest X-ray and bone survey were all normal. Liver function tests were normal, except for an increase in alkaline phosphatase (isoenzyme of liver origin) and bilirubin.

A bone marrow aspirate from the iliac crest demonstrated a mild infiltrate of primitive plasma cells, many of which were multinucleated with prominent nucleoli (fig 1). A needle biopsy of the bone marrow revealed an area of pleomorphic cellular infiltration, mild fibrosis and numerous primitive reticulum cells, some recognizable as Reed-Sternberg cells (fig 2). A lymphangiogram demonstrated enlarged iliac and paraaortic nodes. Laparotomy revealed Hodgkin's disease of the lymphocyte-depleted type involving the liver, spleen (420 g) and retroperitoneal lymph nodes.

The patient was treated with six cycles of nitrogen mustard, vincristine, procarbazine and prednisone (MOPP) at monthly intervals, with immediate resolution of fever, rapid weight gain and return of well-being. After 6 months of therapy he felt normal and the liver function tests had returned to normal. The paraprotein concentration remained unchanged at 1.8 g/100 ml and the sedimentation rate was 60 mm/h. A repeat marrow examination was normal apart from persistence of the plasma cell infiltrate. Free light

chains were now absent from the urine He enjoyed a complete remission from Hodgkin's disease lasting 11 months during which time he received four cycles of MOPP (excluding prednisone), at approximately 2 month intervals as maintenance therapy

In December 1974 he again experienced intermittent fever up to 104°F, rigors, sweats and weight loss Physical examination was normal Hemoglobin 10 2 g/100 ml, white cell count 6,500/µl, platelet count 151,000/µl, sedimentation rate 122 mm/h, total protein 7 4 g/100 ml and albumin 3 8 g/100 ml A bone marrow needle biopsy revealed hypoplastic marrow with mild plasmacytosis and Hodgkin's involvement Treatment with MOPP resulted in only transient fever control and caused significant pancytopenia (platelet count 34,000/µl, white cell count 2500/µl) precluding further therapy Bleomycin, 30 U i v twice a week, and vincristine, 1 5 mg i v weekly, was commenced, together with prednisone 30 mg daily, with rapid relief of symptoms He has remained in remission to the time of writing (4 months since beginning Bleomycin) His latest blood count in April 1975, showed hemoglobin 11 8 g/100 ml, white cell count 9,200/µl and platelet count 360 000/µl The paraprotein level was approximately 1 1 g/100 ml and quantitative immunoglobulin analysis showed IgG 1,770 mg%, IgA 37 mg%, IgM 25 mg%. The patient is being maintained on Bleomycin 10 U and vincristine 1 0 mg weekly i v and 20 mg prednisone by mouth on alternate days

Discussion

It is now well established that there is an increased risk of second neoplasms associated with Hodgkin's disease with an overall incidence of 1 6 to 2 2% [3, 9, 10] In a study of 425 patients with Hodgkin's disease, ARSENEAU *et al* [2] observed a 3 5-fold increase in the risk of developing a second malignancy in all patients, with the greatest risk (29-fold) in a group of 35 patients who had received both intensive radiotherapy and intensive chemotherapy The spectrum of second malignancies in Hodgkin's patients is broad [4, 10, 13], acute leukemia having been particularly emphasized [5, 11, 12, 14] The increased incidence of second neoplasms in Hodgkin's patients has been attributed to the cellular immune deficiency associated with the disease as well as the immunosuppressive effects of radiotherapy and chemotherapy

The occurrence of paraproteinemia in Hodgkin's disease is very rare, having been identified in one study in only 1 of 218 patients [1] Overt myeloma has been documented in association with Hodgkin's disease in three patients previously [4, 7] The patient presented in this report is the first in whom myeloma and Hodgkin's disease were diagnosed simultaneously prior to irradiation therapy or chemotherapy, thus excluding these immunosuppressive modalities from the casual relationship between the two neoplasms The probability that previous immunosuppressive therapy

is not an important factor in the association between Hodgkin's disease and myeloma is further supported by the first case reported by GREENBERG *et al* [7] in whom the interval between irradiation therapy and the diagnosis of florid myeloma could not have been more than 3 months. The diagnosis of myeloma in our patient seemed clearly established by the triad of an IgG-kappa paraprotein, kappa light chains in the urine and myeloma cells in the marrow aspirate. The simultaneous occurrence of Hodgkin's disease and myeloma may represent a chance association of two uncommon malignancies. Alternatively, cellular immune deficiency, as in chronic lymphocytic leukemia and various congenital immunodeficiency diseases [6, 8], may be important in predisposing Hodgkin's patients to an increased risk of developing other neoplasms, including myeloma. There are no reports in the literature of Hodgkin's disease developing at some time after the diagnosis of myeloma, suggesting that it is more likely that Hodgkin's disease predisposes to myeloma than the reverse.

Acknowledgement. Supported by NIH grant AM 16501 and by a Career Research Development Award AM 70551 to Dr. TAVASSOLI.

References

1 ALEXANIAN, R.: Monoclonal gammopathy in lymphoma. Archs. intern. Med. *135*: 62–66 (1975).
2 ARSENEAU, J. C.; SPONZO, R. W.; LEVIN, D. L.; SCHNIPPER, L. E.; BONNER, H.; YOUNG, R. C.; CANELLOS, G. P.; JOHNSON, R. E., and DE VITA, V. T.: Nonlymphomatous malignant tumors complicating Hodgkin's disease. Possible association with intensive therapy. New Engl. J. Med. *287*: 1119–1122 (1972).
3 BERG, J. W.: The incidence of multiple primary cancers. I. Development of further cancers in patients with lymphomas, leukemias and myeloma. J. nat. Cancer Inst. *38*: 741–752 (1967).
4 CAWLEY, J. C.; GOLDSTONE, A. H.; AMNO, J.; REES, J. H. K., and GRANT, A.: Myeloma in a case of Hodgkin's disease. Acta haemat. *52*: 349–355 (1974).
5 CHAN, B. W. B. and MCBRIDE, J. A.: Hodgkin's disease and leukemia. Can. med. Ass. J. *106*: 558–561 (1972).
6 GATTI, R. A. and GOOD, R. A.: Occurrence of malignancy in immunodeficiency diseases: a literature review. Cancer *28*: 89–98 (1971).
7 GREENBERG, B. R.; STATS, D., and GOLDBERG, M.: Simultaneous occurrence of plasma cell multiple myeloma and Hodgkin's disease. N. Y. St. J. Med. *50*: 305–307 (1950).
8 HYMAN, G. A.: Increased incidence of neoplasia in association with chronic lymphocytic leukemia. Scand. J. Haematol. *6*: 99–104 (1969).

chains were now absent from the urine He enjoyed a complete remission from Hodgkin's disease lasting 11 months during which time he received four cycles of MOPP (excluding prednisone), at approximately 2 month intervals as maintenance therapy

In December 1974 he again experienced intermittent fever up to 104°F, rigors, sweats and weight loss Physical examination was normal Hemoglobin 10 2 g/100 ml, white cell count 6,500/μl, platelet count 151,000/μl, sedimentation rate 122 mm/h, total protein 7 4 g/100 ml and albumin 3 8 g/100 ml A bone marrow needle biopsy revealed hypoplastic marrow with mild plasmacytosis and Hodgkin's involvement Treatment with MOPP resulted in only transient fever control and caused significant pancytopenia (platelet count 34,000/μl, white cell count 2500/μl) precluding further therapy Bleomycin, 30 U i v twice a week, and vincristine, 1 5 mg i v weekly, was commenced, together with prednisone 30 mg daily, with rapid relief of symptoms He has remained in remission to the time of writing (4 months since beginning Bleomycin) His latest blood count in April 1975, showed hemoglobin 11 8 g/100 ml, white cell count 9,200/μl and platelet count 360,000/μl The paraprotein level was approximately 1 1 g/100 ml and quantitative immunoglobulin analysis showed IgG 1,770 mg%, IgA 37 mg%, IgM 25 mg% The patient is being maintained on Bleomycin 10 U and vincristine 1 0 mg weekly i v and 20 mg prednisone by mouth on alternate days

Discussion

It is now well established that there is an increased risk of second neoplasms associated with Hodgkin's disease with an overall incidence of 1 6 to 2 2% [3, 9, 10] In a study of 425 patients with Hodgkin's disease, ARSENEAU *et al* [2] observed a 3 5-fold increase in the risk of developing a second malignancy in all patients, with the greatest risk (29-fold) in a group of 35 patients who had received both intensive radiotherapy and intensive chemotherapy The spectrum of second malignancies in Hodgkin's patients is broad [4, 10, 13], acute leukemia having been particularly emphasized [5, 11, 12, 14] The increased incidence of second neoplasms in Hodgkin's patients has been attributed to the cellular immune deficiency associated with the disease as well as the immunosuppressive effects of radiotherapy and chemotherapy

The occurrence of paraproteinemia in Hodgkin's disease is very rare, having been identified in one study in only 1 of 218 patients [1] Overt myeloma has been documented in association with Hodgkin's disease in three patients previously [4, 7] The patient presented in this report is the first in whom myeloma and Hodgkin's disease were diagnosed simultaneously prior to irradiation therapy or chemotherapy, thus excluding these immunosuppressive modalities from the casual relationship between the two neoplasms The probability that previous immunosuppressive therapy

is not an important factor in the association between Hodgkin's disease and myeloma is further supported by the first case reported by GREENBERG *et al* [7] in whom the interval between irradiation therapy and the diagnosis of florid myeloma could not have been more than 3 months. The diagnosis of myeloma in our patient seemed clearly established by the triad of an IgG-kappa paraprotein, kappa light chains in the urine and myeloma cells in the marrow aspirate. The simultaneous occurrence of Hodgkin's disease and myeloma may represent a chance association of two uncommon malignancies. Alternatively, cellular immune deficiency, as in chronic lymphocytic leukemia and various congenital immunodeficiency diseases [6, 8] may be important in predisposing Hodgkin's patients to an increased risk of developing other neoplasms, including myeloma. There are no reports in the literature of Hodgkin's disease developing at some time after the diagnosis of myeloma, suggesting that it is more likely that Hodgkin's disease predisposes to myeloma than the reverse.

Acknowledgement. Supported by NIH grant AM 16501 and by a Career Research Development Award AM 70551 to Dr TAVASSOLI.

References

1 ALEXANIAN, R.: Monoclonal gammopathy in lymphoma. Archs intern. Med. *135:* 62–66 (1975).
2 ARSENEAU, J. C.; SPONZO, R. W.; LEVIN, D. L.; SCHNIPPER, L. E.; BONNER, H.; YOUNG, R. C.; CANELLOS, G. P.; JOHNSON, R. E., and DE VITA, V. T.: Nonlymphomatous malignant tumors complicating Hodgkin's disease. Possible association with intensive therapy. New Engl. J. Med. *287:* 1119–1122 (1972).
3 BERG, J. W.: The incidence of multiple primary cancers. I. Development of further cancers in patients with lymphomas, leukemias and myeloma. J. nat. Cancer Inst. *38:* 741–752 (1967).
4 CAWLEY, J. C.; GOLDSTONE, A. H.; AMESS, J.; REES, J. H. K., and GRANT, A.: Myeloma in a case of Hodgkin's disease. Acta haemat. *52:* 349–355 (1974).
5 CHAN, B. W. B. and MCBRIDE, J. A.: Hodgkin's disease and leukemia. Can. med. Ass. J. *106:* 558–561 (1972).
6 GATTI, R. A. and GOOD, R. A.: Occurrence of malignancy in immunodeficiency diseases: a literature review. Cancer *28:* 89–98 (1971).
7 GREENBERG, B. R.; STATS, D., and GOLDMAN, M.: Simultaneous occurrence of plasma cell multiple myeloma and Hodgkin's disease. N. Y. St. J. Med. *50:* 305–307 (1950).
8 HYMAN, G. A.: Increased incidence of neoplasia in association with chronic lymphocytic leukemia. Scand. J. Haematol. *6:* 99–104 (1969).

9 MOERTEL, C G and HAGEDORN, A B Leukemia or lymphoma and coexistent primary malignant lesions a review of the literature and study of 120 cases Blood *12* 788–803 (1957)
10 RAZIS D V, DIAMOND, H D, and CRAVER L F Hodgkin s disease associated with other malignant tumors and certain non neoplastic diseases Am J med Sci *238* 327–335 (1959)
11 STEINBERG, M H, GEARY, C G, and CROSBY, W H Acute granulocytic leukemia complicating Hodgkin s disease Archs intern Med *125* 496–498 (1970)
12 WEIDEN, P L, LERNER, K G, GERDES, A, HEYWOOD J D, FEFER, A, and THOMAS E D Pancytopenia and leukemia in Hodgkin s disease, report of three cases. Blood *42* 571–578 (1973)
13 WHITELAU, D M Multiple primary carcinomas associated with Hodgkin s disease Can med Ass J *99* 291–294 (1968)
14 AWAAN, F D and SPECK, B *Acute myelomonocytic leukemia in a patient with* Hodgkin's disease Acta haemat *49* 291–299 (1973)

Dr P V SACKS, Scripps Clinic and Research Foundation 476 Prospect Street *La Jolla, CA 92037* (USA)

Acta haemat. 55: 123–128 (1976)

Plasmocytoma with IgM Paraproteinemia. A Case Report

Enzo Fagiolo

Istituto di Clinica Medica, Università Cattolica del Sacro Cuore, Roma

Key Words. Macroglobulinemia · Paraproteins · Plasmocytoma

Abstract. A case of typical diffuse plasmocytoma with IgM paraproteinemia is described. The patient presented with systemic and monomorphic proliferation of atypical and immature plasma cells, osteolytic lesions, retinal venous thrombosis and hemorrhagic diathesis. The therapy with steroids and alkeran produced clinical and hematological remission for 1½ year with a decrease of macroglobulins in the serum. Cell and paraprotein type remained unchanged during the course of the disease. This case, and similar ones already reported in the literature, suggest that neoplastic plasma cell proliferation is accompanied by synthesis of paraproteins including the IgM type. However, this is not characteristic for Waldenström's macroglobulinemia. The author concludes that differential diagnosis in these cases can only be made by cytologic examination.

Proliferating neoplastic plasma cells synthesize 'monoclonal' immunoglobulins of different types (G, A, M, D, E), and/or exclusively light chains. IgM plasmocytoma is very rare, thus differential diagnostic problems with Waldenström's macroglobulinemia might arise. Monoclonal macroglobulinemia has been found in patients showing atypical cytological findings resembling those of Waldenström's cases, and in some patients with typical plasmocytoma. These cases have been differently interpreted and classified. In this paper, a typical case of diffuse plasmocytoma with IgM paraproteinemia, hemorrhagic diathesis and osteolytic lesions is described.

Case Report

Patient M. B., a 53-year-old man, admitted to the Policlinico Gemelli in October 1969 with a 4-month history of recurrent bronchitis, pallor, progressive reduction of

9 MOERTEL, C. G and HAGEDORN, A B Leukemia or lymphoma and coexistent primary malignant lesions a review of the literature and study of 120 cases Blood *12* 788–803 (1957)
10 RAZIS, D V, DIAMOND, H D, and CRAVER, L F Hodgkin's disease associated with other malignant tumors and certain non neoplastic diseases Am J med Sci *238* 327–335 (1959)
11 STEINBERG, M H, GEARY, C G, and CROSBY W H Acute granulocytic leukemia complicating Hodgkin's disease Archs intern Med *125* 496–498 (1970)
12 WEIDEN, P L, LERNER, K G, GERDES A, HEYWOOD, J D, FEFER, A, and THOMAS, E D Pancytopenia and leukemia in Hodgkin s disease, report of three cases Blood *42* 571–578 (1973)
13 WHITELAW, D M Multiple primary carcinomas associated with Hodgkin s disease Can med Ass J *99* 291–294 (1968)
14 AWAAN, F D and SPECK, B Acute myelomonocytic leukemia in a patient with Hodgkin s disease Acta haemat *49* 291–299 (1973)

Dr P V SACKS Scripps Clinic and Research Foundation 476 Prospect Street, *La Jolla, CA 92037* (USA)

Acta haemat. 55: 123–128 (1976)

Plasmocytoma with IgM Paraproteinemia. A Case Report

Enzo Fagiolo

Istituto di Clinica Medica, Università Cattolica del Sacro Cuore, Roma

Key Words. Macroglobulinemia · Paraproteins · Plasmocytoma

Abstract. A case of typical diffuse plasmocytoma with IgM paraproteinemia is described. The patient presented with systemic and monomorphic proliferation of atypical and immature plasma cells, osteolytic lesions, retinal venous thrombosis and hemorrhagic diathesis. The therapy with steroids and alkeran produced clinical and hematological remission for 1½ year with a decrease of macroglobulins in the serum. Cell and paraprotein type remained unchanged during the course of the disease. This case, and similar ones already reported in the literature, suggest that neoplastic plasma cell proliferation is accompanied by synthesis of paraproteins including the IgM type. However, this is not characteristic for Waldenström's macroglobulinemia. The author concludes that differential diagnosis in these cases can only be made by cytologic examination.

Proliferating neoplastic plasma cells synthesize 'monoclonal' immunoglobulins of different types (G, A, M, D, E) and/or exclusively light chains. IgM plasmocytoma is very rare, thus differential diagnostic problems with Waldenström's macroglobulinemia might arise. Monoclonal macroglobulinemia has been found in patients showing atypical cytological findings resembling those of Waldenström's cases, and in some patients with typical plasmocytoma. These cases have been differently interpreted and classified. In this paper, a typical case of diffuse plasmocytoma with IgM paraproteinemia, hemorrhagic diathesis and osteolytic lesions is described.

Case Report

Patient M. R., a 53-year-old man, admitted to the Policlinico Gemelli in October 1969 with a 4-month history of recurrent bronchitis, pallor, progressive reduction of

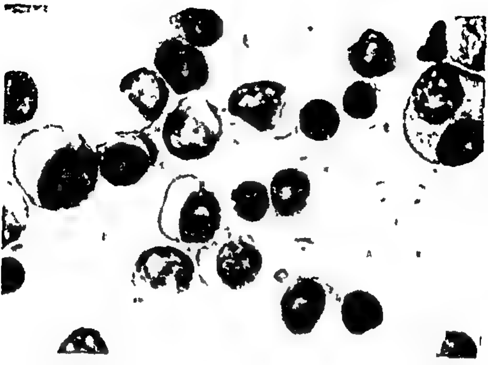


Fig. 1. Sternal marrow: proplasmocytes and plasmoblasts. May-Grunwald-Giemsa. × 800.

vision and repeated epistaxis. On physical examination he presented great pallor and apical systolic murmur. Examination of the fundus revealed bilateral thrombosis of central retinal veins.

Erythrocytes 1 860 000/μl. Hb 4.2 g%; white blood cells 5 800/μl with 40% neutrophils, 53% lymphocytes, 5% monocytes, 2% myelocytes. Erythrocytes presented anisocytosis and rouleaux formation. Platelets 80 000/μl. Sternal puncture showed marked cellularity 70% of which composed mostly by proplasmocytes and by some plasmablasts with light basophilia, generally eccentric nuclei and prominent nucleoli (fig. 1). The normal myeloid cells were altogether scarce. Sedimentation rate: 90 mm/h. Total serum proteins, 11.6%. Serum electrophoresis on cellulose acetate showed: albumin 27%, α_1 globulin 3%, α_2 globulin 6%, β globulin 7%, γ globulin 57%. The γ-peak is of the monoclonal type. The serum immunoelectrophoresis with polyvalent and specific immunoglobulin antisera IgG, IgA, IgM, IgD, IgE showed a very thin but normal IgG and IgA precipitation line, while the IgM line was deformed and very thick (fig. 2). The macroglobulins separated on Sephadex G 200 were formed by light chains of the λ type. Immunoglobulin levels with radial immunodiffusion: IgG 160 mg%, IgA 20 mg%, IgM 4 200 mg%.

Serum ultracentrifugation showed a large fraction (40.5%) of 16.7 S. The serum kept at 4 °C showed a modest amount of cryoglobulin precipitate. The SIA test was positive. There was no proteinuria. Tourniquet test was negative, bleeding time

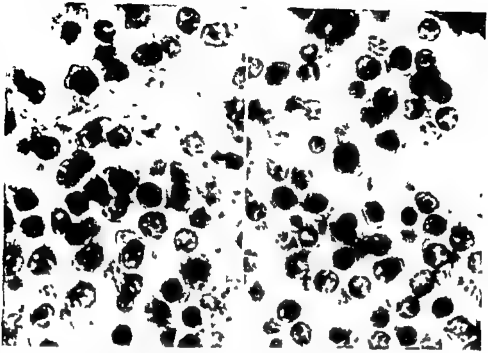
Fig. 2. Immunoelectrophoretic patterns of serum proteins with specific IgM rabbit antiserum

6 min, coagulation time 3 min 30 sec. Quick time 19 sec, prothrombin activity 35%, fibrinogen 70 mg%. Thromboelastogram showed R 24 min and MA 20 mm. Blood urea nitrogen 18 mg%, creatinin clearance 126 ml/min, blood calcium 9.6 mg%, total cholesterol 75 mg%. Complete and incomplete erythrocyte antibodies were absent. Rheuma and Waler Rose tests were negative. X-ray examination of chest bones, vertebral column and skull showed no alterations.

The patient was treated with blood transfusions, prednisone (100 mg/day) and alkeran (8 mg/day). Clinical conditions progressively ameliorated and after several therapy cycles the situation was as follows (October 1970): erythrocytes 4 700 000/µl, Hb 14.3 g%, white blood cells 5 900/µl, platelets 340 000/µl. Total serum proteins, 6.8 g% with 26% of γ-globulins. Immunoglobulin levels: IgG 400 mg%, IgA 13 mg%, IgM 440%. The sternal marrow showed repopulation of normal myeloid cells with 1% plasma cells. The coagulation tests were normal.

In June 1971 the patient commenced to suffer from progressive chest pains, fever, pallor and epistaxis. X-ray examination showed marked osteoporosis of the vertebral column and ribs, conical crush of thoracic vertebrae VI, VII and XI and microalveolar skull structure. The patient was again admitted and treated as previously but his clinical conditions worsened (anemia, bone lesions, hemorrhagic diathesis with epistaxis, hematemesis and melena). In December 1971 the following data were available: erythrocytes 2 600 000/µl, Hb 7.2 g%, white blood cells 4 500/µl, platelets 80 000/µl. Total serum proteins, 12.3 g%. Serum electrophoresis showed 61% γ-globulins with monoclonal peaks. Immunoglobulin levels: IgG 140 mg%, IgA 30 mg%, IgM 1 800 mg%. Immunoelectrophoresis showed a deformed and very thick IgM line. Quick time 15 sec, prothrombin activity 62%, fibrinogen 86 mg%, coagulation time 12 min, bleeding time 5 min. Blood calcium 13 mg%. There was no proteinuria. The sternal marrow was composed almost exclusively by plasma cells.

The patient [died] in February 1972. Autopsy revealed pulmonary edema with bilateral pneumonic foci, turbid myocardosis, gastric and intestinal hemorrhages, splenomegaly with hyperplasia of the red pulp and indistinct Malpighian bodies, plasma cell nodules in the vertebrae and ribs. Histology showed plasma cell infiltrations in vertebrae, ribs, liver, spleen and kidneys (fig. 3).



a b

Fig 3 Histologic patterns monomorphic infiltration of plasma cells in the spleen (a) and the dorsal vertebrae (b) HE. × 400

Discussion

The described case is a diffuse plasmocytoma characterized by (1) *monomorphic proliferation of atypical immature plasma cells in the bone* marrow, liver, spleen and kidneys, (2) osteolytic lesions in the skull, vertebrae and ribs, and (3) hemorrhagic diathesis with prolonged bleeding and coagulation times, reduced prothrombin activity and hypofibrinogenemia An early symptom was reduction of vision due to bilateral thrombosis of central retinal veins Paraproteinemia was of IgM type, λ-light-chains, 16 7 S The cellular aspects and paraprotein pattern remained constant during the course of the disease, although they were quantitatively reduced by steroid and alkeran therapy which induced a $1^1/_2$-year period of clinical and hematological remission

The case presented the typical cellular aspect of plasmocytoma and the paraprotein type of Waldenstrom's macroglobulinemia Difficulty arised for differential diagnosis and nosologic classification since some cases considered as plasmocytoma or plasma cell leukemia with macroglobulin-

emia, presented histologic aspects of the lympho-plasmocytic or lympho-reticulo-plasmocytic type, intermediate between plasmocytoma and Waldenstrom's macroglobulinemia [4, 8, 9, 14]. These cases, however, because of the cellular polymorphism they present are closer to Waldenstrom's disease than to plasmocytoma. It should also be considered that in these cases, it is difficult to establish if the synthesis of 'monoclonal' immunoglobulins is due to neoplastic immunocompetent cells or to immunologically reactive phenomena. Moreover, recent studies have shown the frequency of osteolytic lesions in Waldenström's macroglobulinemia, similar to those found in plasmocytoma [15].

On the other hand, the existence of typical diffuse plasmocytoma with IgM paraproteinemia has been demonstrated [1, 2, 11, 13, 16]. IgG and IgM double paraproteinemia has been recognized in 1 case of diffuse plasmocytoma [10], in liver solitary plasmocytoma [12] and in plasma cell leukemia [6]. IgM paraproteinemia and IgG heavy chains have been found in 1 case of plasma cell leukemia [5]. All these cases, plus the experimental evidence in BALB mice [3, 7], show that neoplastic plasma cells can synthesize IgM as well as the other types of immunoglobulins. Therefore, the presence of a certain immunoglobulin type alone cannot differentiate the various paraproteinemic diseases. The terms 'Waldenström's macroglobulinemia type' or 'myeloma type' paraprotein are thus unsuitable to designate the 'monoclonal' synthesis of IgM or of the other classes of immunoglobulins. Since the presence of IgM paraproteinemia is by no means pathognomonic of Waldenström's disease and since osteolytic lesions are not exclusive of plasmocytoma, differential diagnosis and classification of the different forms of paraproteinemic hemoblastoses can be best made by cytologic examination.

Acknowledgements. The author thanks Dr. Lucia Quercioli (National Red Cross Blood Transfusion Center) for kindly providing ultracentrifugation data.

References

1 Adner, L.; Wallenius, G., and Werner, I.: Macroglobulinemia and myelomatosis. Acta med. scand. *168:* 431–437 (1960).

2 Bureau, Y.; Sinteau, H.; Barrière, H.; Bureau, B.; Litoux, P. et Bray, B.: Macroglobulinémie au cours d'une maladie de Kahler. Presse méd. *76:* 961–964 (1968).

3 BUXBAUM, J and SCHARFF, M D The synthesis, assembly and secretion of gamma globulin by mouse myeloma cells VI Assembly of IgM proteins J exp Med *138* 278–288 (1973)

4 CREYSSEL, R, CHARVILLAT, L, MOREL, P, MATRAY, F, MENDE, S DE ET CROIZAT, P Myélome multiple et dysprotéinémies malignes Revue lyonn Méd *5* 119–171 (1956)

5 KELLER, K, SPLENGLER, G A, SKVARIL, F, FLURY, W, NOSEDA, G und RIVA, G Zur Frage der Heavy chain disease Ein Fall von IgG heavy-chain Fragment und IgM Typ K-Paraproteinämie mit Plasmazellenleukämie Schweiz med Wschr *100* 1012–1022 (1970)

6 MARCHAL, G, FINE, J M et BILSKI PASQUIER, G Macroglobulinémie dans un cas de leucose plasmocytaire Presse méd *70* 425–428 (1962)

7 MCINTIRE, K R, ASOFSKI, R M, POTTER, M, and KUFF, E L Macroglobulin producing plasma cell tumor in mice identification of a new light chain Science N Y *150* 361–363 (1965)

8 MC NUTT, D R and FUDENBERG, H H IgG myeloma with Waldenstrom macroglobulinemia Archs intern Med *131* 731–734 (1973)

9 MOESCHLIN, S Macroglobulinemia Waldenström with miliary lung infiltrations and terminal plasma cell leukemia Acta med scand *445* suppl, pp 154–162 (1966)

10 PENN, G M, KUNKEL, H G, and GREY, H M Sharing of individual antigenic determinants between an γG and a γM protein in the same myeloma serum Proc Soc exp Biol Med *135* 660–665 (1970)

11 PIGNATARO, L, BOLOGNESI, G, MARINONI, G F e RIZZA O Presenza di macroglobulina patologica e fisiologica *in un caso di plasmocitoma* Boll Soc ital Biol sper *29* 228–230 (1962)

12 PRUZANSKI, W, UNDERDOWN, B, SILVER, E H, and KATZ, A Macroglobulinemia myeloma double gammapathy Am J Med *57* 259–266 (1974)

13 RAYNAUD R, BROCHIER M, GRIGUER, P et TADEI A Sur un cas associant la maladie de Kahler et la macroglobulinémie Sang *30* 822–830 (1959)

14 VANNER, J und SIEDENMANN, R Über eine subacut verlaufende osteolytische Form der Makroglobulinämie Waldenstrom mit Plasmazellenleukämie Schweiz med Wschr *87* 1243–1246 (1957)

15 VERMESS M, PEARSON, K D, EINSTEIN, A B, and FAHEY, J L Osseous manifestation in Waldenstrom's macroglobulinemia Radiology *102* 497–504 (1972)

16 WELTON, J WALKER, S R, SCHARP, C, HERENBERG, L A, WISTAR, R, and GREGER, W P Macroglobulinemia with bone destruction Am J Med *44* 280–288 (1968)

Prof ENZO FAGIOLO Istituto di Clinica Medica Università Cattolica del Sacro Cuore, 526, Via della Pineta Sacchetti, *I-00168 Roma* (Italy)

Acta haemat 55 129–139 (1976)

Autologous Marrow Reconstitutions in Severe Aplastic Anaemia after ALG Pretreatment and HL-A Semi-Incompatible Bone Marrow Cell Transfusion[1]

M Jeannet, B Speck, A Rubinstein, B Pelet, M Wyss and H Kummer

Transplantation Immunology Unit, Division of Immunology and Allergy, and Department of Paediatrics, University Hospital, Geneva, Division of Haematology, University Hospital, Basel and Department of Paediatrics, University Hospital Bern

Key Words Antilymphocyte globulin Aplastic anaemia Bone marrow transplantation Marrow reconstitution (autologous)

Abstract Three consecutive patients considered to have end-stage acquired aplastic anaemia were given 100–160 mg/kg antilymphocyte globulin (ALG) i v followed by an infusion of 2–3 8 × 10^8 nucleated marrow cells/kg i v from HL-A one haplotype identical, MLC-positive family donors. All patients showed autologous marrow reconstitutions lasting now 2–3$^1/_2$ years. No clear-cut evidence of marrow engraftment could be established and no graft versus-host disease was seen. It is assumed that these patients had some normal pluripotent haemopoetic stem cells which proved to be able of endoreduplication and of going into cycle after ALG conditioning and allogeneic marrow transfusion

Acquired aplastic anaemia (AA) is a poorly understood entity with a bad prognosis [1–6] Several aetiological factors may be responsible for the disorder, in particular failure of the haemopoetic stem cell, disturbance of the bone marrow matrix, and potentially a disturbance in the humoral factors which regulate haemopoesis [6]

Treatment with anabolic steroids and glucocorticosteroids as well as improved supportive care with antibiotics, platelet and granulocyte transfusions have not markedly improved the prognosis of this disorder [2, 4, 5] Marrow grafting after conditioning with high dose cyclophosphamide

[1] Supported by Swiss National grants for Scientific Research No. 3.8590 72 and 3 0550 73 and the Swiss Cancer League FOR 007.AK 72.

has opened new possibilities for treatment of patients with AA. However, such treatment has so far only been successful if HL-A-identical, mixed lymphocyte culture (MLC)-negative siblings have been available as marrow donors [7]. In HL-A-mismatched situations, fatal graft versus host disease (GvH) has always ensued. Conditioning with heterologous antilymphocyte globulin (ALG) has resulted in split chimaerism in some experimental systems [8, 9] and in several clinical patients [10–12].

However, marrow engarftment was only transient but in a few instances followed by autologous marrow regeneration. GvH was absent despite histoincompatibility in clinical and experimental grafts after this type of conditioning. We are reporting three consecutive patients with long-lasting marrow aplasia who were given marrow cell transfusions from HL-A semi-incompatible, MLC-positive family donors after conditioning with i.v. ALG.

Case Reports

Case 1. A 38-year-old woman was complaining of fatigue, dyspnoea and vaginal bleeding for several months before admission on February 26, 1972. Seven months before this episode, she had received chloramphenicol for the second time in her life, for the treatment of bronchitis. Peripheral blood examination revealed a profound pancytopenia: haematocrit 16%, white cells 1,050 mm³ (neutrophiles 160) and platelets 10,000/mm³. Bone marrow biopsy showed a severely hypoplastic marrow, the cells being mainly lymphocytes. The patient was nursed in a sterile room and received androgens and transfusions for 6 weeks without improvement. She was then given horse antihuman thoracic duct lymphocyte ALG 'Berna' (total dose 150 mg/kg in 5 days). As ALG produces thrombocytopenia, its infusion was regularly followed by intensive platelet transfusion. 24 h after the last dose of ALG she was given 1.2×10^{10} bone marrow cells (2.3×10^{8}/kg) from her HL-A-semi-incompatible, MLC-positive, ABO-identical brother. 2 days later, she developed a general skin eruption with polyarthralgia, cervical lymphadenopathy, and severe pruritus. Abundant plasma cells were noted in the blood smears. She was thought to have serum sickness due to ALG and was started on prednisone. A few days later all symptoms gradually subsided. No signs or symptoms of GvH were observed. The change of cellular blood elements after bone marrow transfusion is illustrated in figure 1.

Reticulocyte and granulocyte counts started to rise about 15 days post grafting. The platelet count improved only slightly, but no bleeding episode was observed during the following weeks. On the 19th day after transplantation, the bone marrow aspirate revealed a marked increase in cellularity. Promyelocytes, myelocytes, numerous foci of normoblasts and occasionally megakaryocytes were found. Blood and platelet transfusions could be discontinued and the patient was discharged on June 1, 1972. During the following months she did well except for mild persisting thrombopenia with vaginal bleeding and an episode of epistaxis. These complica-

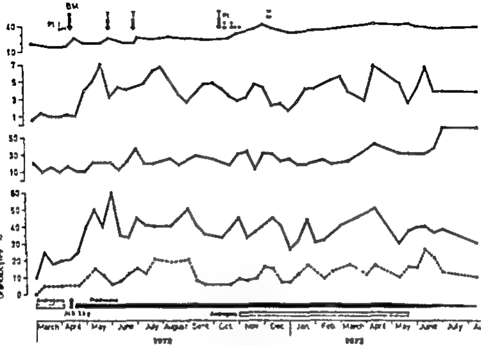


Fig. 1. Haematological events in case 1. BM = Bone marrow transfusion (12×10[10] cells), Pl = platelet transfusion. T = red cell transfusion.

tions were easily controlled by random platelet transfusions. In November 1972 she was restarted on androgens, and prednisone therapy was continued at very low doses (20 mg every other day). In July 1973, the patient was doing well without transfusions, and all drugs were interrupted. In August 1975, over 3 years after bone marrow transfusion, her haematocrit is normal, her granulocyte count is fluctuating around 2,000/mm³ and her platelet count is stable over 100,000/mm³. Androgens and prednisone have been discontinued over 2 years ago. Genetic marker studies, including red cell (N and Le[b]) phaenotypes, HL-A (1, W 32, W 21) antigens, immunoglobulin groups [gm(n) antigens] and Y chromosome, revealed no evidence for engraftment. The function of the patient's T lymphocytes was studied at regular intervals during the post grafting course. As before, the patient's lymphocytes were stimulated by the donor's lymphocytes and by unrelated lymphocytes in MLC. In addition, in the cell-mediated lympholysis (CML) test [13] the patient's lymphocytes were shown, after *in vitro* sensitization, to exert a normal killing effect on ⁵¹Cr labelled target cells carrying the incompatible HL-A antigens of the donor cells. No evidence was found for the presence of a serum factor able to block the MLC or the CML response of the patient's own or of normal lymphocytes [14]. However,

more than 1 year after bone marrow transfusion, a lymphocytotoxic antibody, specific for the incompatible donor antigen HL-A 1, was detected in her serum for the first time. This antibody has persisted since.

Case 2. An 8 year-old boy had recurrent respiratory infections, fatigue and an episode of epistaxis 2 weeks prior to admission to the Childrens' Hospital in Berne on January 3, 1972. Peripheral blood counts gave the following results: haemoglobin 9.1 g%, reticulocytes 0%, leucocytes 730 with 58 neutrophiles/mm³, thrombocytes 200/mm³. Bone marrow aspiration and biopsy revealed a completely aplastic marrow with only small lymphocytes and occasional plasma cells. There was no history of exposure to drugs known to be associated with AA. The patient was nursed in protective isolation and given oxymetholone, prednisone and transfusions. No improvement was seen in the subsequent 5 months, and repeated haemorrhagic and infectious episodes occurred. He progressively became refractory to random platelets, and transfusions usually were associated with febrile reactions. At that time lymphocytotoxic antibodies reactive with 55% of a lymphocyte panel were detected in his serum. ABO and HL-A typing of the family revealed the father to be the best possible marrow donor. As the patient, he had blood group A Rh positive and differed from him, by one HL-A haplotype. In addition the cross match between the patient's serum and the father's lymphocytes was very weak compared to the other family members. In the MLC test, stimulation of the patient's lymphocytes by the father's cells was weaker than with the mother's and with unrelated lymphocytes. ALG was given intravenously on 4 subsequent days (total dose 130 mg/kg). Each dose was followed by a platelet transfusion. 24 h after the last ALG infusion (June 6, 1972), 7×10^9 bone marrow cells (2.3×10^8/kg) from his father were given intravenously. 3 weeks later some myeloid and erythroid precursor cells were detected for the first time in his bone marrow aspirate. Repeated marrow aspirations during the following 6 months revealed an increase of all elements, including megakaryocytes. Granulocyte and reticulocyte counts in the peripheral blood did not show any noteworthy increase before December 1972 (fig. 2). The platelet count was low and he still required frequent platelet transfusions.

However, after the beginning of 1973, when the patient was again on oxymetholone (1.5 mg/kg/day) and prednisone (0.3 mg/kg/day), the transfusion requirement decreased markedly. It is remarkable that after ALG and marrow infusion the patient's refractoriness to random platelets had disappeared. Except for a mild and transient HB Ag negative hepatitis, no infectious or bleeding complications occurred after marrow transfusion. No symptoms typical of GvH were observed. At present, 3 years after grafting, he is back to normal activities. Over more than 1 year he has not had platelet or other transfusions. There is persistent thrombocytopenia of ±10 000/mm³ without major haemorrhagic diathesis. We have planned splenectomy in an attempt to improve this problem. The haemoglobin is normal and granulocyte counts constantly above 2,000/mm³. Genetic markers, including two red cell antigens (Fy^b and Jk^b) and three HL-A antigens (W 10, 2 and 5) disclosed no evidence for marrow engraftment. Twenty serum globulin groups (gm) of the recipient and of the donor were identical, which prevented the detection of a B lymphocyte engraftment. MLC tests performed after transplantation showed an almost complete loss of the reactivity of the patient's lymphocytes to donor lymphocytes treated with mitomycin. In addition, the recipient's serum was found to block the response of his

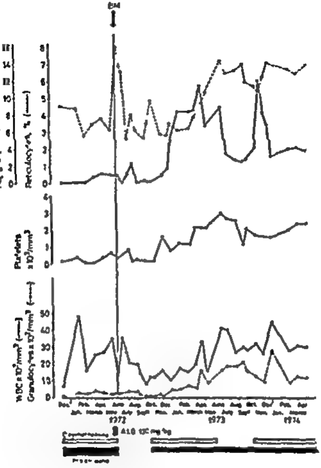


Fig. 2 Haematological events in case 2. BM = Bone marrow transfusion.

own and of normal lymphocytes in allogeneic lymphocytes. A direct CML test [15] performed 9 months after bone marrow infusion showed no killing of donor target lymphocytes by recipient lymphocytes. In the indirect CML test, however, the patient's lymphocytes were shown to have a strong killing effect on donor cells after *in vitro* sensitization.

Case 3. A 12 year-old boy developed haemorrhagic diathesis in September 1970. He was first seen at the Department of Paediatrics of the University of Geneva in November 1970. In the past history, there was no exposure to potentially myelotoxic agents. The haemoglobin was 6.5 g%, leucocytes, 2,600 with 550 neutrophiles per

mm³ and platelets, 27,000 mm³ Bone marrow puncture revealed a severely hypoplastic marrow with predominantly lymphoid and plasmocytoid elements

All other laboratory investigations were normal The diagnosis of idiopathic acquired aplastic anaemia was established and treatment was started with oxymetholone in a dose of 2–3 mg/kg/day and low-dose prednisone After an initial progression of the pancytopenia in the early course of 1971, a partial remission followed which persisted through 1972 The haemoglobin stabilized between 9 and 11 g%, the leucocytes, between 2,000 and 3,000 with 600–1,500 granulocytes/mm³, the platelets, around 20 000/mm³ In Winter 1973, there was clear cut evidence of a relapse which proved to be refractory to androgens and glucocorticosteroids There was haemorrhagic diathesis with profound thrombocytopenia (platelets 1 000–2,000/mm³), progressive leukopenia and granulocytopenia (granulocytes 50–150 mm³) He was given 10 platelet transfusions and a total of 20 units of whole fresh blood and packed red cells At that time, weak lymphocytotoxic antibodies reactive with 30% of a lymphocyte panel were detected in his serum On May 5, 1973, he was transferred to the University Hospital in Basel On admission he had 40 °C fever, generalized petechiae and multiple ulcerations on the oral mucosa. The pancytopenia was progressive with granulocytes of 50/mm³ Bone marrow aspiration and biopsy revealed virtual absence of haemopoetic elements HL-A and ABO typing of the family suggested the mother to be the best marrow donor

The patient was blood group A Rh negative, HL-A 2, W 16 / 2, 12. The mother was A Rh positive, HL-A 2, 12 / 1, W 18 In the patient s serum no lymphocytotoxic antibodies against the mother's lymphocytes were detectable The MLC between donor and recipient was positive The patient was nursed in a sterile laminar cross flow unit He was given sterile food Gastro-intestinal decontamination was achieved with oral nystatin and non absorbable gentamicin and Vancomycin He was conditioned with i v ALG (total dose 160 mg/kg) divided into four doses that were given as 3-hour infusions on 4 subsequent days Six platelet transfusions from 4–6 random donors were necessary to keep his platelet count above 10 000 mm³ during the conditioning period On May 21, 1973, 48 h after the last ALG 1.5×10^{10} nucleated marrow cells from the mother were given i v (3.8×10^{8}/kg) At the end of the marrow infusion, the patient had shaking chills and a temperature of 39 °C) Blood cultures were negative After grafting he was hospitalized for 6 weeks During that period he received only two platelet transfusions on days 8 and 15, then the platelet counts stabilized at 15 000–20 000 mm³ without haemorrhagic diathesis The white cell counts reached 2,500 with 600 granulocytes/mm³ Reticulocyte counts reached 4.3% but there was still a severe anaemia (Hct 12%) at the time of discharge on July 7, 1973 (fig 3) A bone marrow aspiration 3½ weeks after grafting revealed better cellularity, and in particular there was more activity in the myelopoesis where all stages of maturation were found Progenitor cells (CFU C and CFU E) assayed by *in vitro* culture in a methylcellulose system were virtually absent before grafting on stimulation with colony stimulating activity (CSA) and erythropoietin At 3½ weeks after transplantation they were found to be normal in quantity and quality Cytogenetic studies of the marrow cells and of peripheral lymphocytes stimulated with PHA all showed host karyotypes Red cell markers could not be used because of heavy transfusions before grafting and no immunoglobulin allotype markers were available because they were identical between donor and re-

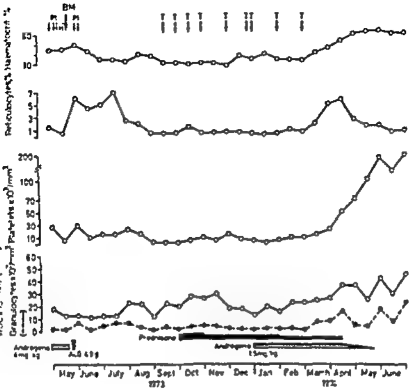


Fig 3 Haematological events in case 3 BM = Bone marrow transfusion (1.5×10^{10} cells) Pl = platelet transfusion T = red cell transfusion.

cipient In October 1973 4 months after bone marrow infusion, the patient was started again on prednisone (0.5 mg/kg day) and 3 months later on oxymetholone (1.5 mg/kg day) In March 1974, his peripheral blood picture improved steadily, and androgens and prednisone were discontinued Since then, there was no more need for any transfusions At present, over 2 years after the graft, he has a normal blood picture with a haemoglobin of 15.9 g/dl, granulocytes above 2 000 mm³, and platelets around 100 000 mm³ For 18 months he has been back to all normal activities The function of the patient's T lymphocytes and B lymphocytes was studied at regular intervals after bone marrow transfusion. As before grafting, the patient's lymphocytes were stimulated by donor lymphocytes in MLC. The level of stimulation increased progressively

Three months after bone marrow infusion, the recipient's serum was found to block the response of his own and normal lymphocytes to allogeneic lymphocytes, a phenomenon which was not observed before At the same time, high titer lymphocy-

totoxic antibodies specific for HL A 5, W 5 and the mother's incompatible antigen W 18 were detected in the patient's serum, demonstrating a strong immunization against the bone marrow donor Since blocking and cytotoxic antibodies appeared almost simultaneously, they may be closely associated A direct CML test [15] performed 6 months after bone marrow infusion showed no killing of donor target lymphocytes by recipient lymphocytes. However, as in the second patient, in the indirect CML test the patient's lymphocytes were demonstrated to have a strong killing effect on donor cells after *in vitro* sensitization

Discussion

The autologous marrow restoration in these three patients suggests that treatment with ALG and HL-A non-identical bone marrow cell transfusion may be of great benefit to patients with severe AA The mechanism of these remissions remains speculative at the moment Spontaneous remissions appear highly unlikely because all patients were considered to have end-stage disease with extreme and long-lasting pancytopenia refractory to conventional therapy All were polytransfused with random blood products In the rabbit with benzene- and ^{32}P-induced AA and in several clinical patients, split and transient haemopoietic engraftments have been achieved after conditioning with ALG followed by marrow infusion [8–12] Despite of histocompatibility differences between donor and recipient, GvH has not been observed Split chimaerism in the rabbit was of a rather constant pattern, donor erythrocytes, granulocytes and B lymphocytes took while T lymphocytes remained of host type [8, 9] In rats rendered aplastic by a lethal dose of cyclophosphamide and treated with rabbit anti rat lymphocyte serum prior to the infusion of incompatible allogeneic bone marrow, no real takes were achieved [16, 17] However, the recipients did not develop GvH and had a significantly prolonged survival compared to the group treated with cyclophosphamide alone A similar beneficial and often more prolonged effect was obtained by the use of allogeneic anti-donor or anti-recipient anti-lymphocyte serum [17] The results in our patients follow the rat model [16, 17] more closely than the rabbit [8, 9] Despite extensive studies, no definite evidence of chimaerism could be demonstrated in our patients Unfortunately, in the second and third one, Ig allotypes could not be used as B-cell markers because of complete identity between donor and recipient The fact that one recipient has produced high titres of specific lymphocytotoxic antibodies against the donor, which were detectable for the first time 1

year after bone marrow infusion, implies that residual donor tissue has persisted for a while in the recipient and has then been rejected. In all three patients it was demonstrated that recipient lymphocytes had a marked capacity of proliferating in MLC with donor cells, and after this phase of sensitization they showed a strong killing effect on them in the CML test. This suggests that no mutual tolerance of donor's and recipient's lymphocytes was established after bone marrow transfusion and confirms the absence of chimaerism. Despite the lack of evidence of engraftments, a pure coincidence between the onset of improvement and the bone marrow graft appears highly unlikely. All three patients had profound granulocytopenia and thrombocytopenia with major haemorrhagic and infectious complications, which are usually associated with a poor prognosis [1, 2, 4, 5]. All had been treated with androgens, the best available supportive care for many months, two without improvement, one with only temporary benefit. Moreover, in all three patients, the rise in marrow cellularity started in a similar chronological and morphological pattern that has been previously reported by other investigators [10–12].

The mechanism responsible for the demonstrated marrow repopulation by host haemopoetic cells remains obscure. It is improbable that ALG alone was operative by suppressing an immune mechanism active at the level of the stem cell or the marrow matrix. Auto-immune phaenomena were searched for in all patients and were all found to be negative. To our knowledge, ALG treatment alone is not beneficial in the treatment of AA. Moreover, ALG reduces the number of colony forming units (CFU) in mouse haemopoetic tissues both *in vivo* and *in vitro* [18]. In other experimental systems, however, it was shown that ALG may stimulate haemopoesis in the spleen [19]. CHERTKOV *et al.* [20] demonstrated in lethally irradiated mice that stimulation of haemopoetic colonization was maximal if ALG was given 24 h prior to the injection of donor haemopoetic cells. It is suggested that pretreatment with ALG may be an important factor for the favourable clinical results in patients with AA given bone marrow transplantations [10–12]. Our results suggest that ALG conditioning and bone marrow grafts may have a synergistic effect. Ultimately a temporary lymphocyte-dependent allogeneic effect [21, 22] may have been responsible for the observed stimulation of recipient haemopoetic tissue. ALG pretreatment might be an essential prerequisite for a local allogeneic effect due to its host immunosuppression allowing split engraftment without overt GvH. The fact that recently two autologous marrow restorations have been observed in patients with severe aplastic

anaemia after conditioning with cyclophosphamide and grafting with *HL-A-identical* sibling marrow [23, 24] implies that other immunosuppressive regimens followed by marrow transfusion can lead to the same results as ALG Irrespective of the underlying mechanism of the results described, we would like to suggest that similar therapeutic trials should be attempted in patients with severe AA who do not have *HL-A-identical* sibling donors

Acknowledgements We wish to thank Prof INDERBITZIN Schweizerisches Serum und Impfinstitut, for supplying the ALG, and Dr N N ISCOVE, F Miescher Institute, Basel, for performing the CFU C and CFU E assays

References

1 LEWIS S M Course and prognosis in aplastic anaemia Br med J *i* 1027–1031 (1965)
2 DAVIS S and RUBIN, A D Treatment and prognosis in aplastic anaemia Lancet *i* 871–873 (1972)
3 KEISER G Erworbene Panmyelopathien Schweiz. med Wschr *100* 1938–1948 (1970)
4 WILLIAMS D M, LYNCH, R E, and CARTWRIGHT, G E Drug induced aplastic anaemia Semin Hemat *10* 195–223 (1973)
5 LI, F P, ALTER B P, and NATHAN, D G The mortality of acquired anaemia in children Blood *40* 153–157 (1972)
6 SPECK, B Toxische Knochenmarkinsuffizienz in Schriftreihe zur Zeitschrift Blut, vol 16 pp 235–245 (1974)
7 STORB R, THOMAS E D, BUCKNER, C D, CLIFT, R A, JOHNSON F L, FEFER, A, GLUCKSBERG H, GIBLETT, E R, LERNER K G and NEIMAN, P Allogeneic marrow grafting for treatment of aplastic anaemia Blood *43* 157–180 (1974)
8 SPECK, B and KISSLING, M Successful bone marrow grafts in experimental aplastic anaemia using antilymphocytic serum for conditioning Eur J biol Res. *16* 1047–1051 (1971)
9 SPECK, B and KISSLING, M Studies on bone marrow transplantation in experimental ^{32}P induced aplastic anaemia after conditioning with antilymphocyte serum Acta haemat *50* 193–199 (1973)
10 MATHÉ, G, AMIEL, J H, SCHWARZENBERG H CHOAY J TROLARD, P SCHNEIDER M, HAYAT, M SCHLUMBERGER J R, and JASMIN, C. Bone marrow graft in man after conditioning by antilymphocytic serum Transplantn Proc *3* 325–332 (1971)
11 MATHÉ G, KIGER, M, FLORENTIN I GARCIA GIRALT E. MARTYRE, M C HALLE PANNENKO O., and SCHWARZENBERG L. Progress in the prevention of GvH bone marrow grafts after ALG conditioning with lymphocyte split chimerism

use of lymphocyte chalone T' and soluble histocompatibility antigens. Transplantn Proc 5 933–939 (1973)
12 Schwarzenberg L. and Mathé, G Bone marrow transplantation after antilymphocyte globulin conditioning split lymphocyte chimerism. Behring Res. Commun ALG therapy and standard zation workshop pp 163–175 (1972)
13 Lightbody J Bernocco D M iogiano V C., and Ceppellini R Cell mediated lympholysis in man after sensitization of effector lymphocytes through mixed lymphocyte cultures G Bact. Virol Immunol 64 243–247 (1971)
14 Jeannet M Rubinstein A., and Pelet B Studies on non HL-A cytotoxic and blocking factor in a patient with immunological deficiency successfully reconstituted by bone marrow transplantation Tissue Antigens 3 411–416 (1973)
15 Garovoy M R Franco V Zschaeck D Carpenter C, B Strom T B., and Merrill J P Direct lymphocyte mediated cytotoxicity as an assay of presensitization Lancet i 573 575 (1973)
16 Jeannet M Vaucher A et Gervasoni C. Prévention de la réaction greffe contre hôte chez le rat après transplantation de moelle osseuse par le sérum antilymphocytaire allogénique Schwe z med Wschr 102 1599–1600 (1972)
17 Werner Ch Ramirez F Gervasoni, C., and Jeannet M Mitigation of graft versus-host disease in rats treated with heterologous and allogeneic antilymphocytic sera Experientia (in press)
18 Meeter T R Anderson N D., and Schaffer C T The effect of heterologous antilymphocyte serum on mouse hemopoetic stem cells. J exp Med. 127 731 745 (1968)
19 Rodriquez Paradisi F Thierfelder S Götze, D Fuliz, M., and Beil, E. Cytology and histology of hemopoetic cell transplantation under the influence of antilymphocyte serum in mice Clin exp Immunol 8 107 119 (1971)
20 Chertkov J L Lemeneva, I N Mendelevitch, O A., and Udalov G A Stimulation of hemopoetic colony formation by antilymphocyte serum. Cell Tiss Kinet 5 357–400 (1972)
21 Rajewsky K Rollants, G E., and Askonas, B A Carrier specificity and allogeneic effect in mice Eur J Immunol 2 592 598 (1972)
22 Kanamaru A Kitamura Y Kawata, T Kanamaru A., and Nagoi K Synergism between lymph node and bone marrow cells for production of granulocytes. I Requirement for immunocompetent cells. Exp Hemat. 2 35 (1974)
23 Speck P Cornu P Jeannet M Nissen C Burri, H F Groff P., Nagel, G A and Buckner C D Autologous marrow recovery following allogeneic marrow transplantation in a patient with severe aplastic anemia. Exp Hemat. (in press)
24 Buckner C D and Thomas, E D personal commun (1975)

PD Dr M Jeannet Hôpital Cantonal Unité d'immunologie de transplantation, 64 avenue de la Roseraie CH 1211 Genève (Switzerland)

Acta haemat 55 140–147 (1976)

Reversible Malabsorption of Folic Acid in the Elderly with Nutritional Folate Deficiency

LARS ELSBORG

Department of Medicine II (Haematology) Aarhus Amtssygehus, and Department of Geriatrics Aarhus Kommunehospital Aarhus

Key Words Folate deficiency Folic acid absorption Geriatric patients Malabsorption Malnutrition

Abstract In normal subjects the folic acid absorption (TRIFA test) was independent of age and sex Among 53 geriatric patients with nutritional folate deficiency, impaired absorption of folic acid was present in 19 (36%) After treatment with folic acid for 4 weeks the absorption returned to normal It is concluded that folate *deficiency per se can produce a malabsorption syndrome resulting in further depletion* of folate Protein deficiency and weight loss alone do not impair the absorption of folic acid

Folate deficiency due to malnutrition is not at all rare in elderly people who because of low income cannot afford a well-balanced diet, or whose dietary choice is restricted because of reduced mobility caused by physical or mental deterioration [13, 17, 19, 20] Low total caloric intake in elderly individuals with low physical activity also reduces the supply of the necessary vitamins [24], and inadequate composition of the diet may lead to depletion of the folate depots within a few months

The study reported here shows that nutritional folate deficiency in elderly *patients can reduce the ability of the intestine to absorb folic acid*

Material and Methods

The 53 patients studied 11 men and 42 women, were all over 60 years (average 74 years) They were admitted to hospital for medical and social problems and had dietetic deficiencies and weight loss (>5 kg) extending over periods from 6 months

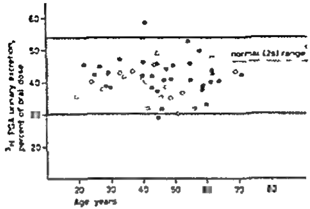


Fig 1 Correlation between results of the TRIFA test, age and sex in 64 normal subjects ● = Male, ○ = female hatched area results obtained in the control group (2s)

to 2 years before admission. Only patients with normal renal function were included in the study

The haemoglobin concentration (normal >7.0 mmol/l) was determined by the cyanhaemoglobin method and the MCV was calculated (normal <100 fl) The serum folate (normal >9 nmol/l) and red-cell folate (normal >250 nmol/l) concentrations were determined by the *Lactobacillus casei* method [11] while the serum cobalamin concentration (normal >200 pmol/l) was determined by an isotope method [15] The formiminoglutamic acid (FIGLU) excretion was measured in 6-hour urine after histidine load (normal <20 μmol/h) Intestinal absorption of folic acid was determined by the TRIFA test [8] The control group for the TRIFA test consisted of 64 randomly selected non folate-deficient subjects aged 23–70 years. Normally more than 30% of the orally administered dose is excreted in the ensuing 24-hour urine

Results

The absorption of folic acid as assessed by the TRIFA test was found to be independent of age and sex in the control group (fig 1)

In the patient group the serum folate concentrations ranged from 0.5 to 9.0 nmol/l (mean 2.8±2.2), while the red-cell folate concentrations varied between 22 and 221 nmol/l (mean 114±67) The FIGLU excretion ranged from 4 to 190 μmol/h (mean 58±50) Haemoglobin levels below 7.0 mmol/l were seen in 25 patients, the MCV was increased in 30 patients The serum cobalamin concentration was normal in all patients

Table I Causes of dietary insufficiency in 53 patients with nutritional folate deficiency

Causes of dietary insufficiency	Males	Females
Faulty dietary habits		6
Abuse of alcohol and medicine	1	5
Forgetfulness, confusion	1	6
Insteady gait (cerebral strokes)		5
Tooth problems		2
Anorexia, neurasthenia	5	4
Loneliness, improper cooking	3	[illegible]
Difficulties of swallowing		2
Hyperthyreosis		4
Undiscovered	1	2
Total	11	42

Table II Absorption of folic acid (TRIFA test) after a 10-day zero-calorie diet

Sex	Age, years	Weight loss, kg	TRIFA, %
M	30	10.0	32.0
F	29	6.8	42.0
F	33	6.7	51.8
F	26	7.2	43.0
F	37	8.2	52.0

except 3, in whom the values were 35, 120 and 150 pmol/l, respectively. None of these patients had pernicious anaemia as assessed by the Schilling test and intrinsic-factor determination in the gastric secretion. Bone marrow studies were performed in 21 patients. Dysplasia of the granulopoiesis as a sign of folate deficiency [1] was demonstrated in 15 patients.

In most of the patients, the cause of the dietary insufficiency could be detected, in some cases, by information given by relatives (table I). Only in 3 patients with distinct evidence of dietary insufficiency was it impossible to trace the cause. In some cases, the patients had placed themselves on an unbalanced diet in the belief that this would have a beneficial effect on colitis, and the caloric requirements were mainly covered by the ingestion of alcohol in others. Owing to confusion and cerebral arteriosclerosis, some patients simply forgot to have their principal meals. In other

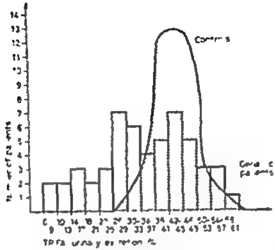


Fig 2 Histogram showing the distribution of the absorption of folic acid (TRI-FA test) in 53 patients with nutritional folate deficiency Hatched area = results obtained in the control group (2 s)

cases, severe physical handicaps restricted the patients' possibilities of doing their shopping of food Often, anorexia had developed in association with a fortuitous infectious disease and had persisted for many months About 20% of the patients were solitary and lived on an unbalanced diet consisting of bread, biscuits and coffee In 4 patients, the weight loss and anorexia were due to thyreotoxicosis.

Determinations of the absorption of folic acid performed by the TRIFA test within the first 3 weeks in hospital gave values below the lower normal limit in 19 patients (fig 2) In the group as a whole, 34 ± 13% of the oral dose of folic acid was excreted in the 24 hour urine, which is significantly different from the values observed in the control group ($p<0.01$)

Having been subjected to the TRIFA test, all patients were treated with an oral dose of 5 mg folic acid three times daily In 11 patients who all showed low absorption of folic acid initially, the TRIFA test was repeated after treatment for 1 month Figure 3 shows that in 9 of these patients, the test then revealed normal values, and in the remaining 2 the absorption had improved although it had not reached the normal level The difference in absorption is significant at the 0 95 level ($p<0.0005$)

to certain organs, as has been observed in conditions with folate deficiency [9, 12, 21, 22]

References

1 BASTRUP MADSEN, P Megaloblastic dysplasia Nord Med *55* 136 (1956)
2 BERG, N O, DAHLQVIST, A, LINDBERG, K, and NORDÉN, A Morphology, dipeptidases and dissacharidases of small intestinal mucosa in vitamin B_{12} and folic acid deficiency Scand J Haematol *9* 167 (1972)
3 BIANCHI, A, CHIPMAN, D W, DRESKIN, A, and ROSENSWEIG N S Nutritional folic acid deficiency with megaloblastic changes in the small bowel epithelium. New Engl J Med *282* 859 (1970)
4 BJURÖ, T Aldringens fysiologi, in HELANDER Gerontologi (Munksgaard, Copenhagen 1974)
5 COOKE, W T, FONE, D J, COX, E V, MEYNELL, M J, and GADDIE, R Acute folic acid deficiency of unknown aetiology temperate sprue Gut *4* 292 (1963)
6 ELSBORG, L Gastrointestinal dysfunction in anticonvulsant megaloblastic anaemia Dan med Bull *15* 180 (1968)
7 ELSBORG, L Folic acid a new approach to its mechanism of intestinal absorption Dan med Bull *21* 1 (1974)
8 ELSBORG, L The folic acid urinary excretion test in the diagnosis of intestinal malabsorption Dan med Bull *20* 102 (1973)
9 ELSBORG, L Multiple jejunal strictures associated with megaloblastic anaemia and myelopathy Acta med scand *196* 127 (1974)
10 FOROOZAN, P and TRIER, J Mucosa of the small intestine in pernicious anemia New Engl J Med *277* 553 (1967)
11 GADE, S, HOFF JØRGENSEN, E, and KRISTENSEN, H P Ø Folinsyrebestemmelse i serum og erythrocytter Ugeskr Læg *134* 2473 (1972)
12 GRANT, H C, HOFFBRAND, A V, and WELLS, D G Folate deficiency and neurological disease Lancet *II* 762 (1965)
13 HANSEN, H A and NYSTRÖM, B Blood folic acid levels and folic acid clearance in geriatric cases Geront clin *3* 173 (1961)
14 HÄGERSTAD, M Studies of conjugated folates in nutrition and their absorption, thesis, Lund (1974)
15 KARSTOFT, H Assay of serum vitamin B_{12} using an isotope method. The Schilling test. Scand J Gastroenterol *9* suppl 29, pp 33–38 (1974)
16 KLIPSTEIN, F A, LIPTON, S D, and SCHENK, E A Folate deficiency of the intestinal mucosa Am J clin Nutr *26* 728 (1973)
17 MOLLIN, D L and WATERS, A H Nutritional megaloblastic anaemia in BLIX Occurences, causes and prevention of nutritional anaemias Swedish Nutrition Foundation Symposium vol 6, pp 121–134 (Uppsala 1968)
18 MONTGOMERY, R D, BEALE, D J, SAMMONS, H G, and SCHNEIDER, R Postinfective malabsorption A sprue syndrome Br med J *II* 265 (1973)
19 MORGAN, A G, KELLEHER, J E, WALKER, B E, LOSOWSKY, M S, DROLLER H, and MIDDLETON, R S W A nutritional survey in the elderly Haematological aspects Int Z VitamForsch *43* 461 (1973)

20 Read, A. E., Gough, K. R., Pardoe, J. L., and Nicholas, A.: Nutritional studies on the entrants to an old people's home, with particular reference to folic acid deficiency. Br. med. J. ii: 843–848 (1965).
21 Reynolds, E.H.; Rothfeld, P., and Pincus, J. H.: Neurological disease associated with folate deficiency. Br. med. J. ii: 398 (1973).
22 Strachan, R. W. and Henderson, J. G.: Dementia and folate deficiency. Q. Jl Med. 142: 189 (1967).
23 Veeger, W.; Ten Thije, O. J.; Hellemans, N.; Mandema, E., and Nieweg, H. O.: Sprue with a characteristic lesion of the small intestine associated with folic acid deficiency. Acta med. scand. 177: 493 (1965).
24 Wretlind, A.: Näringsläget hos den svenska befolkningen. In Reizenstein: Klinisk nutrition (Stockholm 1968).
25 Wildenhoff, K. E. and Sørensen, N. S.: Absolut faste i behandling af adipositas. Nord. Med. 82: 1197 (1969).

Dr. I. Elsborg, Med. Department II, Aarhus Amtssygehus, DK-8000 Aarhus C (Denmark)

Acta haemat. 55: 148–152 (1976)

Proliferative Activity of Undifferentiated Cells (Blast Cells) in Preleukaemia

M. FISCHER, P. S. MITROU and K. HÜBNER[1]

Zentrum der Inneren Medizin, Abteilung für Hämatologie
(Leiter: Prof. Dr. med. H. MARTIN) und Senckenbergisches Zentrum der Pathologie
Abteilung III (Leiter: Prof. Dr. med. K. HÜBNER),
Johann Wolfgang-Goethe Universität, Frankfurt a.M.

Key Words. Autoradiography · Blast cells · Cytophotometry · DNA synthesis · Preleukaemia

Abstract. Undifferentiated cells (blast cells) in preleukaemic states show labelling indices comparable to those of leukaemic blast cells. Since this cell population can remain constant over months or even years, a steady state must exist between the production of blast cells and cell death. It is more likely, however, that in preleukaemic conditions blast cells may differentiate to morphologic 'normal' haemopoietic cells. The fact that the latter show an impaired cell proliferation supports the view that they derive from an affected stem cell compartment.

Undifferentiated cells (blast cells) can occur in the bone marrow, more seldom in the bone marrow and blood of patients with bicytopenia or pancytopenia. The number of blast cells can remain constant over several months or even years. They differ in this from the blast cells of acute leukaemia, the number of which increases continuously.

Leukaemic blasts have, as opposed to normal haemopoiesis, a reduced proliferative activity and a prolonged generation time [2, 3, 5, 7, 8, 10–12, 14]. Their labelling index is variable and, for example, dependent on the degree of the leukaemic infiltration [3, 8, 12, 14]. The less the density of the blast cells in the marrow, the higher is the labelling index in general. Accordingly, in a so-called smoldering acute leukaemia a higher labelling index was found than in acute myeloblastic leukaemia [13].

[1] We wish to thank Miss M. BROSZITS for her excellent technical assistance.

In our investigations on ineffective haemopoiesis in preleukaemic states [4, and unpub.] we also examined the small blast cell population, specifically with the question whether blast cells in preleukaemia always show a higher labelling index, corresponding to the slight marrow infiltration, than leukaemic blast cells.

Methods

Single cell suspensions of bone marrow were incubated for 1 h at room temperature with tritiated thymidine (2 μCi/ml, specific activity 5 Ci/mM). Smears were stained by the May-Grünwald-Giemsa method. Cells were photographed and the cell areas marked with an 'Objektmarker' (Leitz, FRG) on a second slide. The panchromatic stain was leached out and then the smears were restained by the Feulgen method (hydrolysis in 1 n HCl at 60 °C for 9 min). The DNA content was measured with a cytophotometer UMSP 1 (Zeiss, FRG) at 560 nm. Autoradiographs were prepared by the dipping method using the emulsion K5 (Ilford, England) as described previously [4].

Results

The undifferentiated cells (blast cells) of the bone marrow of 5 patients were examined. The most important haematologic data are summarized in table I. In the first examination, all patients had pancytopenia or bicytopenia with normal or increased cell density of the bone marrow. The percentage of blast cells of the bone marrow accounted for maximally 15%. In the peripheral blood blast cells were only detectable in two patients. In three of them, the complete picture of an acute leukaemia appeared after a few months.

The labelling index of the blast cells (table II) was 4.5–10% in 4 of 5 cases, only in 1 case was the index very low with 2%. In comparison to the number of blast cells in DNA synthesis, a relatively high number of unlabelled cells has a DNA content between 2 c and 4 c (U cells).

Discussion

The labelling indices of the undifferentiated cells (blast cells) of preleukaemia are comparable to those of leukaemic blasts [5, 7, 8, 10–12]. It has, however, been shown that the percentage of DNA synthesis of leukaemic blasts is smaller the denser the leukaemic marrow infiltration is at

Table I Main haematological findings in the patients examined

se	Age years	Hb g/100 ml	Neutro-phils/µl	Platelets × 10³/µl	Blast cells/µl blood	Blast cells in bone marrow, %	Ratio E G	Diagnosis	Course
	54	12 0	210	16	0	4	1 0	leukopenia and thrombopenia	haematolog c ta unchanged months) Bact infections
	48	8 7	2 740	97	571	15	0 2	smoldering acute leukaemia?	died 6 month. diagnosis AM
	59	6 5	1 110	120	0	6	0 25	anaemia and leu kopenia hyper-plastic bone mar-row (Preleukaemia?)	died 4 months diagnosis AM
	23	5 2	384	17	24	7	1 0	pancytopenia with hyperplastic bone marrow Early stage of AML?	blood transfu Haematolog c ta unchanged (6 months)
	33	8 5	360	87	0	10	0 4	pancytopenia with hyperplastic bone marrow (Preleukaemia?)	died 3 months diagnosis AMI

Ratio E G = Ratio erythropoiesis myelopoiesis The percentage of the blast cells refers to all the ce bone marrow in the assessment of 200 cells

the time of the examination [8, 12, 14] One would therefore expect more blast cells in DNA synthesis in our patients than in cases with dense leukaemic infiltration In spite of the small number of blast cells in our 5 patients, their labelling index is low A further difference to the blasts of acute myeloblastic leukaemia is the slight or even absent tendency to increase in numbers Many patients with the diagnosis 'preleukaemia' or smoldering acute leukaemia' can have a constant number of blast cells in the bone marrow over many months or even years It is not possible to give an exact explanation of this phenomenon The following hypotheses present themselves (1) the lifetime of the blast cells formed is short, they die intra medullarly Production and death of blast cells are therefore, in a steady state, (2) blast cells can differentiate to cells of the 'normal' hae

Table II Results of the cytophotometric-autoradiographic examination

Case No	n	G_1	S	G_2	U
1	153	81 6	8 5	0 7	9 2
2	209	91 4	4.3	0 5	3 8
3	202	88 1	9 9	1 0	1 0
4	89	85.4	10 1	1 1	3 4
5	193	97 0	2.0	0	2.0

n = Number of cells examined U = unknown, unlabelled cells with DNA values between the diploid and tetraploid content

mopoiesis [6, 9] This seems to be possible, at least from the morphological point of view, because the 'normal' haemopoiesis and, above all, the myelopoiesis of our patients show abnormalities with numerous transitional forms between blast cells and promyelocytes or myelocytes Our cytophotometric autoradiographic investigations on myelopoiesis of these cases show a highly disturbed proliferation with a decreased labelling index and a high percentage of cells in the presynthetic phase (unpublished results) For example in patient 5, 98% of all promyelocytes and myelocytes are found in the G_1-phase and only 2% had incorporated ^3H thymidine These cells do not differ in this in their proliferative activity from the blast cells We might, therefore, assume that the morphologically undifferentiated cells (blast cells) are progenitors of haemopoiesis or of a part of haemopoiesis [9] This hypothesis is supported by the finding of HOELZER *et al* [6] that myeloblasts may differentiate in *diffusion chambers* Furthermore, the demonstration of the same chromosomal aberrations in blast cells and erythroblasts in cases of acute leukaemia shows that 'normal' erythropoiesis is also affected [1] In preleukaemic states the reduced proliferative activity of the haemopoiesis indicates that *'normal' myelopoiesis and erythropoiesis* derive from an affected stem cell compartment

References

1 BLACKSTOCK, A. M and GARSON, O M Direct evidence for involvement of erythroid cells in acute myeloblastic leukaemia. Lancet *ii* 1178 1179 (1974)

Table I Main haematological findings in the patients examined

Case No	Age years	Hb g/100 ml	Neutro-phils/μl	Platelets ×10³/μl	Blast cells/μl blood	Blast cells in bone marrow, %	Ratio E G	Diagnosis	Course
1	54	12 0	210	16	0	4	1 0	leukopenia and thrombopenia	haemato ta uncha months) infection.
2	48	8 7	2.740	97	571	15	0 2	smoldering acute leukaemia?	died 6 m diagnosis
3	59	6 5	1 110	120	0	6	0 25	anaemia and leukopenia, hyper-plastic bone marrow (Preleukaemia?)	died 4 m diagnosis
4	23	5 2	384	17	24	7	1 0	pancytopenia with hyperplastic bone marrow Early stage of AML?	blood tr Haemato ta unchai (6 month
5	33	8 5	360	87	0	10	0 4	pancytopenia with hyperplastic bone marrow (Preleukaemia?)	died 3 m diagnosis

Ratio E G = Ratio erythropoiesis myelopoiesis The percentage of the blast cells refers to all the bone marrow in the assessment of 200 cells

the time of the examination [8, 12, 14] One would therefore expect mo blast cells in DNA synthesis in our patients than in cases with der leukaemic infiltration In spite of the small number of blast cells in our patients, *their labelling index is low*. *A further difference to the blasts* acute myeloblastic leukaemia is the slight or even absent tendency increase in numbers Many patients with the diagnosis 'preleukaemia' smoldering acute leukaemia' can have a constant number of blast cells the bone marrow over many months or even years It is not possible give an exact explanation of this phenomenon The following hypothe present themselves (1) the lifetime of the blast cells formed is short, th die intra medullarly Production and death of blast cells are, therefore, a steady state, (2) blast cells can differentiate to cells of the 'normal' ha

Table II Results of the cytophotometric-autoradiographic examination

Case No	n	G_1	S	G_2	U
1	153	81.6	8.5	0.7	9.2
2	209	91.4	4.3	0.5	3.8
3	202	88.1	9.9	1.0	1.0
4	89	85.4	10.1	1.1	3.4
5	193	97.0	2.0	0	2.0

n = Number of cells examined, U = unknown, unlabelled cells with DNA values between the diploid and tetraploid content

mopoiesis [6, 9] This seems to be possible, at least from the morphological point of view, because the 'normal' haemopoiesis and, above all, the myelopoiesis of our patients show abnormalities with numerous transitional forms between blast cells and promyelocytes or myelocytes Our cytophotometric-autoradiographic investigations on myelopoiesis of these cases show a highly disturbed proliferation with a decreased labelling index and a high percentage of cells in the presynthetic phase (unpublished results) For example in patient 5, 98% of all promyelocytes and myelocytes are found in the G_1-phase and only 2% had incorporated ^{3}H-thymidine These cells do not differ in this in their proliferative activity from the blast cells We might, therefore, assume that the morphologically undifferentiated cells (blast cells) are progenitors of haemopoiesis or of a part of haemopoiesis [9] This hypothesis is supported by the finding of HOELZER *et al* [6] that myeloblasts may differentiate in diffusion chambers Furthermore, the demonstration of the same chromosomal aberrations in blast cells and erythroblasts in cases of acute leukaemia shows that 'normal' erythropoiesis is also affected [1] In preleukaemic states, the reduced proliferative activity of the haemopoiesis indicates that normal myelopoiesis and erythropoiesis derive from an affected stem cell compartment

References

1 BLACKSTOCK, A. M and GARSON, O M Direct evidence for involvement of erythroid cells in acute myeloblastic leukaemia. Lancet *i*: 1178–1179 (1974)

2 Clarkson, B, Strife, A, Fried, J, Sakai, Y, Ota, K., Ohkita, T., and Masuda, R. Studies of cellular proliferation in human leukemia IV Behavior of normal hematopoietic cells in 3 adults with acute leukemia given continuous infusions of ³H thymidine for 8 or 10 days. Cancer 26 1–19 (1970)

3 Cronkite, E. P Kinetics of leukemic cell proliferation Seminars Hematol 4 415–423 (1967)

4 Fischer, M, Mitrou, P S., and Hübner, K Proliferation of erythroblasts in refractory anaemia. A combined autoradiographic study Acta haemat 52 257–264 (1974)

5 Hale, A J and Cooper, E. H DNA synthesis in infectious mononucleosis and acute leukaemia Acta haemat 29 257–266 (1963)

6 Hoelzer, D, Kurle, E und Harris, E. B Leukämische Erkrankungen der Granulopoese Störungen eines Regelkreises. 3 Int. Arbeitstag über Proliferative Erkrankungen des myeloischen Systems, Wien 1975

7 Killmann, S A Proliferative activity of blast cells in leukemia and myelofibrosis Acta med scand 178 263–280 (1965)

8 Killmann, S A Acute leukemia the kinetics of leukemic blast cells in man An analytical review Ser haemat 3 38–102 (1968)

9 Killmann, S A Acute leukemia Development remission/relapse pattern, relationship between normal and leukemic haemopoiesis, and the 'sleeper to feeder stem cell hypothesis Ser haemat. 3 103–128 (1968)

10 Mauer, A M and Fisher, V Characteristics of cell proliferation in four patients with untreated acute leukemia Blood 28 428–445 (1966)

11 Mauri C., Torelli U, Artusi T, Grossi, G und Emilia G Autoradiographische Untersuchungen über den RNA und Proteinstoffwechsel der unreifen Zellen bei akuter Leukämie Schweiz. med Wschr 95 1485–1487 (1965)

12 Pileri A, Gabutti, V, Masera P., and Gavosto, F Proliferative activity of the cells of acute leukemia in relapse and in steady state Acta haemat 38 193–199 (1967)

13 Queisser U, Ohlischläger A, Queisser, W., and Hempel, H Cell proliferation in the 'preleukemic' phase of acute leukemia Acta haemat 47 21–32 (1972)

14 Schmidt, J R, Kiely, J M, Tauxe, W N., and Owen, C A jr Cell proliferation in leukemia during relapse and remission Acta haemat 36 313–322 (1966)

Dr M Fischer, *Abteilung für Hämatologie Zentrum für Innere Medizin* Theodor Stern Kai 7 *D-6000 Frankfurt a.M* (FRG)

Acta haemat 55 153-159 (1976)

Low Mitotic Activity in Eosinophilic Leukaemia

U Sjögren

Department of Internal Medicine, University Hospital Lund

Key Words Bone marrow eosinophils · Eosinophilia · Eosinophilic leukaemia · Leukaemia · Mitotic index

Abstract The mitotic indices of the eosinophilic precursors in the bone marrow of 3 untreated patients with eosinophilic leukaemia were found to be significantly lower than those of 11 patients with reactive eosinophilia and those of 15 healthy controls The same differences were found in the neutrophilic precursors and in the myeloblasts indicating leukaemic involvement of both the eosinophilic and neutrophilic cell lines Mitotic countings therefore seem to be of diagnostic significance in this rare disease

The separation of eosinophilic leukaemia (EL) from other conditions with eosinophilia is difficult. Furthermore, there are at least two different types of EL (a) acute EL with a marked number of myeloblasts (MB) in the peripheral blood and immature eosinophils dominating in the bone marrow (patients K S and E R. in this paper), and (b) chronic EL with increased numbers of mostly mature eosinophils in the blood (patient T M) [3 7, 9, 10, 19] Although it is quite clear that leukaemic cells are rather heterogenous with respect to their kinetic behaviour, low mitotic indices of the granulopoietic precursor cells, especially the MB, indicate leukaemic involvement of these cells Thus, significantly lower indices have been found in acute myeloid leukaemia [6, 15, 28, 29] and in chronic myeloid leukaemia especially in the more advanced cases [2, 5, 21, 26-28]

The aim of the present work was to investigate whether any characteristic changes in mitotic activity of the MB, the neutrophilic precursors

and the eosinophilic precursors will occur in cases of EL, thus, making the diagnosis surer

Material and Methods

Patients Three patients with EL, 11 patients with reactive eosinophilia (RE) and 15 haematologically healthy persons were included in this study None of the patients had received steroids or cytostatics

EL group The first patient, T M., a 52 year old man was studied on December 4, 1974 Hb was 5 7 g/dl, WBC 24 300 with 72% mature eosinophils and 2% MB thrombocytes 162,000 The patient had splenomegaly and increasing WBC Thus, on March 25, 1975 the WBC were 183 000 with 88% eosinophils The LAP score was negative A detailed case report with a chromosome analysis is given by MITTELMAN *et al* [18] The chromosome study revealed no Ph¹-chromosome but an isochromosome 17 in all bone marrow metaphases The patient is still alive

The second patient K S, a 65 year old man, was studied on June 27, 1972 Hb was 4 9 g/dl WBC 32,500 with 2% immature eosinophils 41% MB and 14% myelocytes thrombocytes only 1 000 VAMP treatment was not successful and the patient died on November 11, 1972 Autopsy was not performed

The third patient E R a 61 year old woman was studied on April 4 1974 Hb was 8 3 g/dl WBC 15 500 with 4% immature eosinophils and 55% MB thrombocytes 35 000 Treatment with Cytosine arabinoside and Rubidomucin has been rather successful and the patient is alive Bone marrow data for the patients above are given in table I

RE group 11 patients with diagnoses such as asthma allergic reaction helminthic diseases Hodgkin's disease and dermatitis herpetiformis were studied The bone marrow eosinophils comprised 6 4–39.3% of all nucleated cells and in the peripheral blood 29–64% were eosinophils Hb was 10.5–14.2 g/dl and WBC 4 600–28 600

Normals 15 probands without perceivable haematologic disorders and with clinical diagnoses such as spondylolisthesis cervical disc prolapse adipositas psychiatric disorders and unverified hypoginadismus served as controls Hb 12,1–16 6 g/dl and WBC 2,200–8,300 In the bone marrow the eosinophils comprised 1 4–5 1%

Bone marrow examination The bone marrow smears were stained with May Grünwald Giemsa Through examination of 1 000–2,500 (mean 1 094) nucleated cells the percentages of granulocytic cells at different stages of maturation were determined The cells were classified according to HEILMEYER and BEGEMANN [13] and SJÖGREN [26] A cell was considered to be in mitosis from the stage of partial loss of nuclear membrane in prophase until nuclear reconstitution had appeared at the end of the telophase The granulocytic precursor cells and their mitoses are described below

Myeloblasts The diameter varies from 12 to 20 μm but sometimes smaller ones micromyeloblasts, are found. The nucleus with 2–6 light blue nucleoli is round or oval and with finer chromatin than the pronormoblasts. The nucleus occupies most of the cell and the nuclear border is distinctly but delicately drawn There are no

Table I Bone marrow data from 3 patients with EL (values give percentage of all nucleated cells)

Patient	MB	NPMC	NMC	EPMC	EMC	Eosinophils at all	Remarks
T M	2.2	5.4	13.6	11.6	18.9	53.9	'nuclear flags', mixed baso-eosinophilic granules
[redacted] S	16.2	19.4	4.9	4.0	10.8	34.7	vacuoles in the cytoplasm mixed baso-eosinophilic granules
E. R	22.7	3.5	0.4	15.3	17.7	57.7	

granules in the narrow basophilic cytoplasm In the mitotic phase the chromosome formation occupies most of the cell

Neutrophilic (NPMC) and eosinophilic (EPMC) promyelocytes Diameter 18–25 μm The nucleus is large with visible nucleoli. The cytoplasm is basophilic The granules of the NPMC are large and azurophilic The EPMC have a mixture of azurophilic and eosinophilic granules and sometimes there are large violet granules too especially in the leukaemia cases The chromosomes are similar to those in the MB

Neutrophilic (NMC) and eosinophilic (EMC) myelocytes The cells are smaller than in the PMC (14–20 μm) The nucleus with a coarse chromatin structure and no nucleoli is rounded or kidney-shaped and is often situated eccentrically The granulation of the cytoplasm is specifically neutrophilic or eosinophilic The mitotic figures are comparatively small with thick chromosomes.

Mitotic Indices

EL group In the 3 patients, 858 11 042 and 4,500 MB respectively were counted and 6 33 and 56 mitoses respectively, were found 6900 17 040 and 1 150 NPMC+NMC respectively were counted and 31 62 and 6 mitoses respectively were found 10 903 10 058 and 6,540 EPMC+EMC respectively, were counted and 83 44 and 36 mitoses respectively were found

RE group 190–1 261 (totally 7 481) MB were counted and 5–27 (totally 161) mitoses were found 1 476–7,890 (totally 52,326) NPMC+NMC were counted and 20–101 (totally 613) mitoses were found. 1 040–5 016 (totally 28,254) EPMC+EMC were counted and 13–67 (totally 358) mitoses were found

Normals 33–191 (totally 1 137) MB were counted and 27 mitoses were found. 1 316–3 614 (totally 30 445) NPMC+NMC were counted and 13–43 (totally 348) mitoses were found 17 214 (totally 1,576) EPMC+EMC were counted and 19 mitoses were found

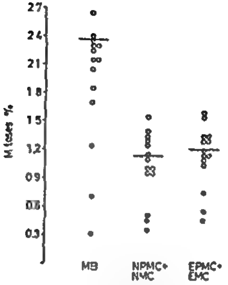


Fig 1 Separate mitotic indices of MB, NPMC+NMC and EPMC+EMC in 3 patients with EL (●) and in 11 patients with RE (O) Horizontal lines indicate mean value of 15 normals

Because of the nuclear and cytoplasmatic asynchrony of neutrophilic and eosinophilic precursors in the EL group it was very difficult to separate the promyelocytic and myelocytic mitoses. Therefore the PMC and MC were pooled into one group

Results

The mitotic indices of the MB in the EL group were significantly lower than those of the MB in the RE group (Mann-Whitney two-tailed U test, $p<0.02$) (fig 1) The mitotic indices of the NPMC+NMC in the EL group were significantly lower ($p<0.02$) than those of the corresponding cells in the RE group The mitotic indices of the EPMC+EMC in the EL group were significantly lower than those of the EPMC+EMC in the RE group ($p<0.02$) (fig 1) In the EL group 12–27% of the eosinophils were EPMC and 31–35% EMC In the RE group 5–11% (median 7) were EPMC and 18–45% (median 28) were EMC. In the normals 3–12% (median 5) were EPMC and 26–43% (median 34) were EMC There is a significantly higher proportion of EPMC in the leukaemia group compared to the RE group and to the normals The proportions of eosinophilic precursors (EPMC+EMC) within the eosinophilic pool of the RE patients

and the normals are in good agreement with data by GROSS and GEDIGK [11] who found 37% EPMC+EMC in normal subjects

Discussion

There has always been some controversy regarding the classification of EL in the group of true leukaemias and some authors have considered it doubtful that this rare disease is a clinical entity [4, 20, 22, 31, 33] Various abnormalities of the eosinophils in EL have been recorded such as nuclear-cytoplasmatic asynchrony, abnormally large eosinophilic granules, mixed basoeosinophilic granules and vacuoles in the cytoplasm [1, 3, 7, 20, 30, 32, 33], but there is no proof that similar changes do not occur in dieseases with benign eosinophilia. Karyotype analyses have shown chromosome abnormalities in some cases of EL [8, 10, 12, 14, 17, 18, 25] but only in a very few cases the aberrations can be referred to certain cell types, e g both eosinophils and neutrophils [12, 17, 18] Leukaemic involvement of both these cell lines has also been proposed in morphologic and histochemical investigations [1, 19] If we accept the idea of the MB as the precursor of both the eosinophils and the neutrophils it may be useful to study the kinetic behaviour of the MB separately As mentioned before low mitotic indices of the MB is probably pathognomonic for the myeloid leukaemias Separate mitotic countings of the NPMC+NMC and the EPMC+EMC would give information of the extension of the leukaemic cell population as well Low mitotic indices of the EPMC+EMC in combination with dominance of morphologically abnormal eosinophils in the bone marrow may then justify the diagnosis of EL. All the 3 patients reported here fulfilled these criterias The median values for the mitotic indices of the EPMC+EMC were 0.55% in the EL group and 1 27% in the RE group This difference cannot be the result of various proportions of PMC in the two groups since there were more PMC, normally known to have higher mitotic indices than the MC [16, 23, 24] in the EL group Very little is known about the mitotic activity of the eosinophils in various conditions with eosinophilia A very high mitotic index of 2.3% of the EPMC+EMC is reported by GROSS and GEDIGK [11] and in two cases of chronic myeloid leukaemia the corresponding indices were 1 24 and 1 39% respectively, i e the same as those of the NPMC+NMC [2] In this paper the mitotic indices of the eosinophils were slightly higher than those of the neutrophils in all 3 patient groups Thus the mitotic

activity of the EPMC+EMC in cases of RE and in normals could be estimated to be 1 0–1 6% and significantly lower in cases of EL.

Acknowledgement I wish to thank Dr MAJ LUNDSTRÖM, of Stockholm who brought the third EL patient to my attention and supplied me with bone marrow smears

References

1 ACKERMAN G A Eosinophilic leukemia A morphologic and histochemical study Blood *24* 372–388 (1964)
2 BACCARANI, M and KILLMANN, S A Cytokinetic studies in chronic myeloid leukaemia Evidence for early presence of abnormal myeloblasts Scand J Haematol 9 283–292 (1972)
3 BENVENISTI, D S and ULTMANN, J E Eosinophilic leukemia Ann intern Med *71* 731–745 (1969)
4 BOUSSER, J , Eosinophilie et leucémie Sang *28* 553–560 (1957)
5 BRANDT L , MITELMAN, F , NILSSON, P G , and SJÖGREN, U Low mitotic activity of granulopoietic precursor cells in Ph¹-chromosome negative chronic myeloid leukaemia Lancet *ii* 719 (1974)
6 BRANDT, L., MITELMAN, F , and SJÖGREN, U Relation between chromosomes and mitotic activity in acute myeloid leukemia Hereditas 79 305–306 (1975)
7 DAMESHEK, W and GUNZ, F Leukemia, 2d ed., pp 226-230 (Grune & Stratton, New York 1964)
8 ELVES M V and ISRAËLS, M C. G Cytogenetic studies in unusual forms of chronic myeloid leukaemia Acta haemat. *38* 129–141 (1967)
9 EVANS, T S and NESBIT, R. R Eosinophilic leukemia Blood *4* 603–613 (1949)
10 GOH, K., SWISHER, S N , and ROSENBERG, C. A Cytogenetic studies in eosinophilic leukemia Ann intern Med *62* 80–86 (1965)
11 GROSS R und GEDIGK, P Eosinophilie im Knochenmark, in BRAUNSTEINER Physiologie und Physiopathologie der weissen Blutzellen p 22 (Thieme Stuttgart 1959)
12 GRUENWALD, H , KIOSSOGLOU, K. A , MITUS, W J , and DAMESHEK, W Philadelphia chromosome in eosinophilic leukemia Am J Med *39* 1003–1010 (1965)
13 HEILMEYER L. und BEGEMANN H Atlas der klinischen Hämatologie und Cytologie (*Springer, Berlin 1955*)
14 KAUER, G L and ENGLE, R L Eosinophilic leukaemia with Ph¹ positive cells Lancet *ii* 1340 (1964)
15 KILLMANN, S-A. Acute leukemia The kinetics of the leukemic blast cells in man An analytical review Ser Haemat Sect I *3* 38–102 (1968)
16 KILLMANN S-A , CRONKITE, E P , FLIEDNER T M., and BOND V P Mitotic indices of human bone marrow cells I Number and cytologic distribution of mitoses Blood *19* 743–750 (1962)

17 Kiossoglou K. A., Mitus, W. J., and Dameshek, W. Cytogenetic studies in the chronic myeloproliferative syndrome. Blood *28* 241–252 (1966)

18 Mitelman, F., Panani, A., and Brandt, L. Isochromosome 17 in a case of eosinophilic leukaemia. An abnormality common to eosinophilic and neutrophilic cells. Scand. J. Haematol. *14* 308–312 (1975)

19 Morelli A. e Maurea, C. Leucemia eosinofila. Haematologica *37* 435–497 (1953)

20 Odeberg B. Eosinophilic leukemia and disseminated eosinophilic collagen disease – a disease entity? Acta med. scand. *177* 129–144 (1965)

21 Ogawa, M., Fried J., Sakai Y., Strife, A., and Clarkson B. D. Studies of cellular proliferation in human leukemia. VI. The proliferative activity, generation time and emergence time of neutrophilic granulocytes in chronic granulocytic leukemia. Cancer *25* 1031–1049 (1970)

22 Rickles F. R. and Miller D. R. Eosinophilic leukemoid reaction. J. Pediat. *80* 418–428 (1972)

23 Rondanelli, E. G., Magliulo E., Giraldi, A., and Carcò F. F. The chronology of the mitotic cycle of human granulocytopoietic cells. Blood *30* 557–568 (1967)

24 Salera U. e Tamburino G. Studio dell'attività proliferativa dei granuloblasti. Haematologica *37* 1135–1167 (1953)

25 Sandberg A. A., Ishihara, T., Crosswhite, L. H., and Hauschka, T. S. Comparison of chromosome constitution in chronic myelocytic leukemia and other myeloproliferative disorders. Blood *20* 393–423 (1962)

26 Sjögren U. Morphologic studies of haemopoietic tissue in myeloid leukaemias and megaloblastic anaemia. thesis Lund (1975)

27 Sjögren U., Brandt, L., and Mitelman F. Relation between life expectancy and composition of the bone marrow at diagnosis of chronic myeloid leukaemia. Scand. J. Haematol. *12* 369–373 (1974)

28 Tsuchiya J. and Maekawa, T. Cytokinetic studies on hematopoietic cells of the bone marrow. Acta haemat. *36* 641–660 (1973)

29 Urasiński I. Mitotic index in various forms of acute leukaemia in the light of own investigations. Acta haemat. *4* 19–26 (1973)

30 Weinger R. S. André-Schwartz, J. Desforges, J. F., and Baker M. Acute leukaemia with eosinophilia or acute eosinophilic leukaemia. A dilemma. Br. J. Haemat. *30* 65–70 (1975)

31 Wolkowa M. A. To the question of the eosinophilic leukaemia. Klin. Med., Mosk. *52* 50–55 (1974)

32 Yam L. T., Li, C. Y., Necheles, T. F., and Katayama, I. Pseudoeosinophilia, eosinophilic endocarditis and eosinophilic leukemia. Am. J. Med. *53* 193–202 (1972)

33 Zucker-Franklin D. Eosinophilia of unknown etiology. A diagnostic dilemma. Hosp. Pract. *6* 119–127 (1971)

Dr. U. Sjögren, Department of Internal Medicine, University Hospital, *S-221 85 Lund* (Sweden)

Acta haemat. 55: 160–168 (1976)

Receptor Studies on 19 Cases of Non-Hodgkin Malignant Lymphocytic Lymphoma

A. E. STUART and J. A. HABESHAW

Department of Pathology, University of Edinburgh Medical School, Edinburgh

Key Words. Antibodies · Cell receptors · Immunology · Lymphocytes · Malignant lymphoma

Abstract. The receptor profile of 19 cases of non Hodgkin lymphocytic lymphoma has been described. In nodular lymphomas, the values commonly fall within normal limits. In 2 cases, the nodules separated from the paranodular tissue contained a proportion of sheep-erythrocyte-rosetting cells as well as fluorescent cells. The diffuse lymphomas show predominance of fluorescent cells but some contain numerous B and T cells. These represent 'co-operative' neoplasms. In 3 cases, a receptor profile characterized by high frequency of surface fluorescence and a low incidence of IgG and EAG receptors was noted; this may represent a pattern for a new subclass of malignant lymphoma. Tumour cells rich in surface fluorescence contained abundant ribosomes. Receptor silence was noted only in a single case.

Current attempts to understand the nature of lymphoid neoplasms postulate that these tumours represent an abnormal immunological response. The introduction of antigen is normally followed by a proliferation of macrophages and various populations of lymphocytes. Early stages of this response are characterized by the participation of antigen handling cells and later by effector cells which either synthesize Ig or are agents of cell mediated immunity. At critical points in this cellular sequence the reacting system may be frozen, for reasons unknown, in attitudes of continued cellular proliferation [8]. The presence of recognisable receptors on the surface of neoplastic cells has facilitated the study of the cellular composition of lymphoid tumours. Thus a malignant mediastinal lymphoma (Sternberg sarcoma) has been shown as thymic in origin [9], follicular lymphomas have a B cell origin [4] and 10 cases of non-Hodgkin lymphoma have been classified as

B or T cell neoplasms [7] The present report attempts to correlate morphology with functional studies in a series of 10 cases of non-Hodgkin malignant lymphocytic lymphoma

Materials and Methods

Lymph nodes were collected fresh at operation, and immediately transferred to Hank's balanced salt solution, or medium 199 in a sterile container at room temperature The node and container were then placed in melting ice for conveyance to the laboratory On arrival, nodes were sectioned into 2 mm slices, and a portion fixed for histology The remaining node was minced into small fragments, or teased in medium 199 containing heparin 5 units/ml, to produce a cell suspension The suspension was filtered through 80-mesh stainless steel gauze to remove cell clumps Viability was assessed by trypan blue dye exclusion, and smears of the suspension were made The cell suspension was washed 3 times in medium 199, and cells were counted

Surface receptor determination The techniques employed here have been reported in detail elsewhere [5]

Sheep-erythrocyte-rosetting cells Washed fresh red blood cells from a single sheep were used throughout Lymph node cells and sheep red blood cells were mixed in a ratio of 1 40, incubated for 5 min at 37°C, centrifuged at 120 *g* for 5 min and incubated for 2 h at 4°C Serum-free medium 199 was used throughout Rosettes were suspended by gentle rotation of tubes in the long axis, sealed under a coverslip and counted wet By this method 50–60% of normal peripheral blood mononuclears form rosettes with more than 3 erythrocytes

Receptors for immune complex (erythrocyte+antibody IgG EAIgG) The test particle was the sheep erythrocyte coated with a high subagglutinating dose of rabbit anti-sheep erythrocyte IgG Lymph node cells and sensitized sheep erythrocytes were mixed in a ratio of 1 40, incubated for 10 min at 20°C, centrifuged at 120 *g* for 5 min, and incubated for 2 h at 4°C. Rosettes were resuspended by vigorous end-over-end rotation of the tube

Receptors for complement (erythrocyte+antibody+complement EAC) Sheep erythrocytes were sensitized by incubation with a high subagglutinating dose of rabbit anti-sheep erythrocyte IgM antibody for 1 h at 37°C. They were then treated with fresh human serum or guinea pig complement (Wellcome) using a two-stage procedure previously described [5] The presence of complement on the sensitized cells was shown by their agglutinability with anti-C3 antiserum (Meloy) they were not agglutinated by anti-C4 Rosettes were prepared by mixing lymph node cells with sensitized cells in a ratio of 1 40 incubating for 10 min at 37°C. They were then centrifuged at 120 *g* for 5 min and incubated for 2 h at 4°C Rosettes were suspended by vigorous end-over-end rotation of the tube Antibody sensitized cells alone gave low background rosettes with normal peripheral blood lymphocytes (0.2–4 4%) and similar numbers of rosettes were obtained at 4°C incubation (mean value 23%) as at 37°C (mean value 21%) with complement-sensitized cells These cells formed rosettes with most surface Ig+ lymphocytes, and with a high proportion of glass-adherent cells from normal peripheral blood

Neutral red test Phagocytic cells were detected by their characteristic pattern of con

centration of neutral red dye when incubated in dilute solution of dye in phosphate-buffered saline

Surface-Ig-carrying cells Cells carrying surface Ig were detected by a sandwich technique using polyvalent rabbit anti human Ig antiserum, followed by reaction with goat anti rabbit antiserum coupled with fluorescein isothiocyanate (FITC conjugate) Aggregates were removed by high-speed centrifugation before use. All viable cells showing detectable surface fluorescence were counted Fluorescent capping cells showed clear caps, or confluent ring fluorescence, fluorescent non-capping cells were classified as mononuclear cells showing small discrete dots of fluorescence diffusely localized on the cell surface

Separation of nodules from nodular lymphomas Nodules were separated from the spleen of case 6, and a lymph node from case 9 In both cases the nodules were 'washed free of loosely adherent cells by threefold sedimentation through medium 199 at 1 *g* The nodules were then disrupted by squeezing through 80-mesh stainless steel gauze

Histology The tumours were classified as nodular or diffuse, and the degree of differentiation assessed in terms of cell size, nuclear regularity, amount of cytoplasm and prominence of nucleoli The Rappaport classification was used but in the diffuse lymphomas a need was felt for a category of 'intermediate differentiation' [1] Cases 1, 2, 3, 6, 7 in the diffuse lymphomas were classed as poorly differentiated, the remained as intermediate The nodular lymphomas correspond to the Rappaport poorly differentiated type Histiocytic lymphomas were rigorously excluded from both series

Results

Lymph nodes removed from patients without signs of lymphoreticular disease showed a wide variation in receptor pattern (table I) Total surface-Ig-positive cells never exceeded 50% and the mean number was 29% Higher values were recorded for T cells which gave a mean of 43% Proportions of cells bearing receptors for IgG or complement-sensitized erythrocytes bore no constant relationship either to immunofluorescent cells (B cells) or sheep-erythrocyte-rosetting cells (T cells) in lymph node cell suspensions

The three pathological but non-neoplastic nodes gave unusual values (table II) Case 1 was from a patient with toxoplasmosis and histology showed numerous large germinal centres A high proportion of surface-Ig-positive cells was present, the number of IgG- and EAC-rosetting cells was relatively low Case 2 was a lipomelanic reticulosis containing an excess of plasma cells, about 30% 'receptor-silent' cells were present Case 3 was a chronic non specific lymphadenitis from a patient with dermatitis Marked paracortical hyperplasia was present and the percentage of T lymphocytes was increased

Table I Lymph nodes from 10 cases without overt lymph node disease

Case No	Viability	SRBC rosettes	IgG rosettes	EAC rosettes	Fluorescent capping cells	Fluorescent non-capping cells	Fluorescent total cells	Neutral red phagocytes
	%	%	%	%	%	%	%	%
1	[illegible]	59	26	15	29	5	34	5
2	[illegible]	48	56	36	[illegible]	3	16	20
3	[illegible]	41	8	–	14	8	22	–
4	[illegible]	24	12	–	30	8	38	–
5	80	38	13	52	26	9	35	27
6	65	55	[illegible]	40	28	4	32	27
7	84	51	7	5	8	4	12	11
8	74	35	29	33	[illegible]	2	34	7
9	[illegible]	32	11	24	10	[illegible]	48	16
10	93	50	45	–	13	9	22	–
Mean ± SD	82±8	43±11	23±17	29±16	20±9	9±11	29±11	16±9
Range	65–93	24–59	7–56	5–52	8–32	2 38	12–48	5–27

Viab l ty is expressed as a percentage of viable cells in the lymph node suspension The different classes of lymph node cells are also expressed as a percentage of cells in suspens on.

Table II Lymph nodes from 3 cases with non-neoplastic lymphadenopathy

Case No	Diagnosis	Viabil ty	SRBC rosettes	IgG rosettes	EAC rosettes	Fluorescent capping cells	Fluorescent non-capping cells	Fluorescent total cells	Neutral red phagocytes
		%	%	%	%	%	%	%	%
1	Toxoplasmosis	78	15	7	2	47	3	50	7
2	Lipomelanic reticulosis	90	34	6	6	18	3	21	9
3	Non-specific lymphaden tis	[illegible]	52	9	ND	16	5	21	8
Mean ± SD		79±11	35±17	7±2	4±3	27±17	4±1	31±17	8±1
Range		65 90	[illegible] 52	6–9	2–6	16–47	3 5	[illegible] 50	7–9

Table III Lymph nodes from 8 cases of nodular lymphocytic lymphoma

Case No	Viability %	SRBC rosettes %	IgG rosettes %	EAC rosettes %	Fluorescent capping cells %	Fluorescent non-capping cells %	Fluorescent total cells %	Neutral red phagocytes %
1	90	59	9	12	–	–	–	5
2	90	52	41	–	31	10	41	6
3	80	48	23	29	23	12	35	1
4	71	41	21	44	30	25	55	2
5	73	30	20	15	35	4	39	8
6 spleen	90	42	38	27	32	10	42	4
7	69	8	6	4	38	27	65	17
8	75	46	67	8	31	9	40	6
Mean ± SD	80±9	41±16	28±20	20±14	31±5	14±9	45±11	6±5
Range	69–90	8–59	6–67	4–44	23–38	4–27	35–65	1–17

Table IV R supraclavicular node of nodular lymphoma case 9

	Paranodular tissue %	Nodule, %
SRBC rosettes	27	21
EAIgG rosettes	25	22
EAC rosettes	32	5
Neutral red phagocytes	1	2
Fluorescent capping cells	14	26
Fluorescent non-capping cells	57	64

Viability nodule 80% cells viable paranodular tissue 76 5% cells viable

The results in 8 cases of nodular lymphocytic lymphoma (table III) show the majority of cases have a receptor pattern within normal limits Case 6 underwent a staging laparotomy, the spleen contained multiple small lymphomatous nodules in which both sheep-erythrocyte rosetting cells and fluorescent cells were present in approximately equal numbers Case 7 had a preponderance of fluorescent cells and unusually low percentages of IgG and EAC-rosetting cells Case 8 had a high percentage of IgG rosettes suggesting that this receptor can be expressed both by T and B cells Case 9

Table V Lymph nodes from 11 cases of diffuse lymphocytic lymphoma

Case No	Viability %	SRBC rosettes %	IgG rosettes %	EAC rosettes %	Fluorescent capping cells %	Fluorescent non-capping cells %	Fluorescent total cells %	Neutral red phagocytes %
1	84	23	23	17	57	37	94	6
2	[illegible]	8	6	3			74	1
3	88	6	55	–	36	34	70	2
4	80	14		–	47	17	59	–
5	93	14	4	4			53	1
6	91	[illegible]	58	50	15	27	42	18
7	85	64	11	12			3	4
8	[illegible]	38	23	22	3	30	33	4
9	90	40	40		43	7	50	–
10		14	17	21	1	2	3	3
11	67	26	8	7	38	2	40	2
Mean ± SD	84 ± 9	26 ± 18	26 ± 21	17 ± 15	29 ± 21	20 ± [illegible]	47 ± 28	5 ± 5
Range	62 93	6–64	4 58	3 50	1 57	2 37	3–94	1 18

(table IV) contained 21% of sheep-erythrocyte rosetting cells within the nodules and 90% of cells were fluorescent

The receptor pattern in 11 cases of diffuse lymphoma (table V) is different from the nodular variety Cases 1–5 show a predominance of fluorescent cells indicating a B cell origin The presence of low numbers of IgG and EAC receptor bearing cells in association with a high percentage of surface Ig positive cells (case 2) is again noted Case 7 was the only one showing a predominance of T cells Case 9 is important because unequivocal histology revealed a uniform and homogenous picture in which equal numbers of T and B cells were recorded Receptor silence was present in case 10 Case 11 represents a repeat biopsy from case 1 of the nodular lymphoma series where the histology had changed from the nodular to the diffuse form Low numbers of cells expressing IgG and EAC receptors were found in both biopsies

Discussion

The receptor pattern of our 8 cases of nodular lymphoma fall largely within normal limits similar findings were reported by JAFFE *et al* [4] These

5 HABESHAW, J A and YOUNG, G Quantitation of subclasses of mononuclear cells in normal human blood by membrane receptor studies Br J Haemat 29 43 (1975)
6 HABESHAW, J A and STUART, A E Cell receptor studies on seven cases of diffuse histiocytic malignant lymphoma (reticulum cell sarcoma) J clin Path 28 289 (1975)
7 PETER, C R, MACKENZIE M R, and GLASSY, F J T or B cell origin of some non Hodgkin s lymphomas Lancet ii 686 (1974)
8 SALMON, S E and SELIGMANN, M B cell neoplasia in man Lancet ii 1230 (1974)
9 SMITH J L, BARKER, C R, CLEIN, G P, and COLLINS, R C Characterisation of malignant mediastinal lymphoid neoplasm (Sternberg sarcoma) as thymic in origin Lancet i 74 (1973)
10 STEIN, H, LENNERT, K, and PARWARESCH, M R Malignant lymphomas of B-cell type Lancet ii 855 (1972)

Dr J A HABESHAW, Department of Pathology, University Medical School Teviot Place *Edinburgh EH8 9AG* (Scotland)

Acta haemat. 55: 169–173 (1976)

Acute Disseminated Lymphosarcoma with B Cell Markers in a Child

P. MOSCATELLI, F. DAGNA BRICARELLI and A. RASORE QUARTINO

Chair of Human Genetics of the University and Pediatric Department of the Galliera Hospital (Head: Prof. G. SANSONE), Genoa

Key Words. B cell markers · Immunology of malignant lymphoma · Lymphosarcoma · Meningeal leukemia

Abstract. A case of a 4-year-old child with disseminated lymphosarcoma with meningeal involvement is described. Immunological investigations of the blast cells in the cerebrospinal fluid were carried out and in most of them Ig determinants and no response to PHA stimulation were found. So the B-dependent nature of these cells was ascertained. This observation is a contribution to the diagnosis of lymphoid malignancies through the evaluation of the immunological nature of the blast cells. The data of the literature are still scarce and inconclusive.

In this study, the B dependent nature of the cells of a disseminated lymphosarcoma occurring in a 4-year old child is demonstrated by the lack of response to PHA and by the presence of Ig receptors in nearly all the cerebrospinal fluid (CSF) cells available during a meningeal invasion.

Case Report

The patient is a 4-year-old boy first seen in our Department on November 27, 1972. A short time before, he had had a small, hard swelling in the left supraorbital region. A biopsy was made and sent to us.

On admission, the physical examination showed only pre-auricular and submandibular lymphadenopathy. Red blood cell count: 4.2 million/µl, hematocrit: 38%, hemoglobin: 11.8 g%, platelet count: 270 000/µl, white blood cell count: 5 000/µl with 49% neutrophils, 47% small lymphocytes, and 4% monocytes. ESR, serum Ig, blood urea nitrogen, serum electrolytes, urinary catecholamines, a bone marrow aspirate, skeleton X-rays were all within normal limits.

5 Habeshaw, J. A. and Young, G.: Quantitation of subclasses of mononuclear cells in normal human blood by membrane marker studies. Br. J. Haemat. [illegible] (197[illegible]).
6 Habeshaw, J. A. and Stuart, A. E.: Cell receptor studies on seven cases of diffuse histiocytic malignant lymphoma (reticulum cell sarcoma). J. clin. Path. [illegible] (197[illegible]).
7 Payne, C. R.; MacKenzie, M. R.; and Cleave, F. L.: T or B cell origin of some non-Hodgkin's lymphomas. Lancet ii: [illegible] (197[illegible]).
8 Seligmann, [illegible] and [illegible]: B cell neoplasia in man. Lancet [illegible] (197[illegible]).
9 Stein, [illegible]; [illegible]; [illegible]; and [illegible]: Characterization of malignant [illegible] lymphoid [illegible]. Lancet [illegible] (197[illegible]).
10 Stein, H.; Lennert, K.; and Parwaresch, M. R.: Malignant lymphomas of B-cell type. Lancet ii: [illegible] (197[illegible]).

Dr. J. A. Habeshaw, Department of Pathology, University Medical School, Teviot Place, Edinburgh EH8 9AG (Scotland)

Acta haemat. 55 169-173 (1976)

Acute Disseminated Lymphosarcoma with B Cell Markers in a Child

P. MOSCATELLI, F. DAGNA BRICARELLI and A. RASORE QUARTINO

Chair of Human Genetics of the University and Pediatric Department of the Galliera Hospital (Head: Prof. B. SANSONE), Genoa

Key Words. B cell markers · Immunology of malignant lymphoma · Lymphosarcoma · Meningeal leukemia

Abstract. A case of a 4-year-old child with disseminated lymphosarcoma with meningeal involvement is described. Immunological investigations of the blast cells in the cerebrospinal fluid were carried out and in most of them Ig determinants and no response to PHA stimulation were found. So the B-dependent nature of these cells was ascertained. This observation is a contribution to the diagnosis of lymphoid malignancies through the evaluation of the immunological nature of the blast cells. The data of the literature are still scarce and inconclusive.

In this study, the B dependent nature of the cells of a disseminated lymphosarcoma occurring in a 4-year-old child is demonstrated by the lack of response to PHA and by the presence of Ig receptors in nearly all the cerebrospinal fluid (CSF) cells available during a meningeal invasion.

Case Report

The patient is a 4-year-old boy first seen in our Department on November 27, 1972. A short time before, he had had a small, hard swelling in the left supraorbital region. A biopsy was made and sent to us.

On admission, the physical examination showed only pre-auricular and submandibular lymphadenopathy. Red blood cell count: 4.2 million/µl; hematocrit: 38%; hemoglobin: 11.8 g%; platelet count: 270,000/µl; white blood cell count: 5,000/µl with 49% neutrophils, 47% small lymphocytes, and 4% monocytes. ESR, serum Ig, blood urea nitrogen, serum electrolytes, urinary catecholamines, a bone marrow aspirate, skeleton X-rays were all within normal limits.

In the above-mentioned biopsy, striated muscular tissue and adipose tissue were heavily infiltrated by monomorphous cells showing round nuclei with a dense chromatin pattern and a scarcely visible and coarse cytoplasm, sometimes appearing as a halo. An infiltration of immature lymphoid cells was diagnosed.

A diagnosis of regional lymphosarcoma was then made and antiblastic therapy started. Vincristine (1.5 mg/m²) was administered on a weekly schedule and cyclophosphamide (100 mg/m²) daily, while a radiotherapy course of 3,000 R was completed on the site of the biopsy. The boy was treated as an outpatient until June 1973, when he was admitted again because of increasing pallor. Red blood cell count was then 3 million/µl, hematocrit, 25%, hemoglobin, 7.8 g%. A bone marrow aspirate showed a nearly complete substitution of the normal cell population by atypical lymphoid cells. A diagnosis of disseminated lymphosarcoma was made and a more aggressive antiblastic polychemotherapy started (thioguanine, rubidomycine, arabinoside cytosine, methotrexate, prednisone, cyclophosphamide, vincristine: TRAMPCO, every 2 weeks). A complete remission was promptly obtained: on September 18, a bone marrow aspirate revealed a normal cell population. The cyclic polychemotherapy was continued and the child was well until March 1974, when he suddenly presented with headache, vomiting and strabismus. The physical examination showed: mydriasis, reduced light reflex of the pupils, right 6th and left 7th nerve palsy, impaired swallowing reflex, bilateral Babinski's sign, cranial tympanism and other symptoms of meningeal irritation. A funduscopic examination, EEG and skull X rays confirmed the clinical diagnosis of increased intracranial pressure. A lumbar puncture showed an increased pressure of the CSF which contained a small number of blasts (36/µl). A therapeutic regimen including cranial irradiation (^{60}Co, 2,400 R in a 4-week period) and intrathecal methotrexate (7.5 mg/m² weekly) was established. Symptoms of meningeal involvement rapidly improved and now, after 8 months, the child is in marrow remission and his spinal fluid appears normal. He is still on intrathecal methotrexate and TRAMPCO.

Immunological investigations. CSF cells and peripheral blood lymphocytes, separated on a Ficoll Hypaque gradient, were tested against an anti human globulin (IgG+IgA+IgM) antiserum labeled with fluorescein isothiocyanate (Behringwerke). The response rate to *in vitro* stimulation with PHA was also evaluated. Most of the CSF lymphoid cells showed Ig determinants (80%) and a lack of response to PHA stimulation. In peripheral blood an impaired phytoresponsiveness and an increased rate of fluorescent cells have been observed (65%) (table I), the latter results being in sharp contrast with the low values of serum Ig (IgG: 400 mg%, IgA: 75 mg%, IgM: 30 mg%). As CSF cells were so scarce, immunofluorescence tests could not be performed either with monospecific or with anti κ- and anti λ-chain sera.

Discussion

The results clearly demonstrate the B-dependent nature of the blast cells. The study of the surface markers of lymphoid cells is of greatest usefulness not only for understanding normal immunity, but also in the analysis of neoplasia affecting the lymphopoietic system [12]. As for pediatric patients

Table I

Source of the cells	B lymphocytes, %	Control cpm	PHA M 0.01 ml/ml cpm	PHA/control
CSF	80	2,610 ± 450	4,080 ± 931	1.56
Peripheral blood	53	3,060 ± 339	49,575 ± 9,026	16.20

Control = unstimulated lymphocytes, PHA M: phytohemagglutinin M-(Difco) stimulated lymphocytes, cpm: counts per minute (results are expressed in cpm as the mean value of replicate cultures after incubation with ³H thymidine), $\frac{\text{PHA}}{\text{control}}$: ratio of stimulated to unstimulated lymphocytes

only, pathological cells of subjects with acute lymphocytic leukemia (ALL) have been found to possess in general impaired markers either for B or T cells [2, 4, 8, 14, 16, 18]. Recently, however, cases of ALL have been reported, in which blast cells were able to bind sheep erythrocytes or to react with antisera to thymocytes, therefore suggesting involvment of T cells [3, 10–12, 14]. Most of the above mentioned investigations have been done on CSF cells because only in such conditions no contamination from normal cells occurs.

The immunological characteristics of blasts in childhood malignant lymphomas are scarcely known, but, with a single exception, a T derivation seems to be demonstrated in the cases described untill now [5, 9, 11]. The only exception was a 14-year-old patient, described by GAIL-PECZALSKA *et al.* [7] who developed leukemia during an abdominal lymphoma. The presence of Ig molecules on the surface of his blast cells clearly suggested an involvment of the B dependent system. KAPLAN *et al.* [9] reported 4 cases whose cells – a suspension of tumor or CSF cells collected during meningeal involvement – had a very high rate of E rosette forming cells and an almost complete absence of receptors for membrane Ig and complement (EAC rosette). A T derivation has been looked upon by CASTLEBERRY *et al.* [5] in 2 cases of lymphosarcoma who first developed acute leukemia and subsequently CNS involvement. More than 99% of the cells reacted with antisera prepared with lymphoid cells of agammaglobulinemic patients or with fetal thymocytes. This is in contrast with the observations of adult lymphosarcomas which are frequently of B-dependent nature [1, 6, 17].

Our data are complementary to the previous reports of the pediatric literature. It is evident that identical possibilities of B or T involvement exist if malignant lymphoma has to be considered as a cancer of the lymphoid system [9].

The practical and theoretical interest of the study of lymphocyte surface markers has been recently emphasized by KAPLAN *et al.* [9] who point out its usefulness in the differential diagnosis between ALL and malignant lymphomas which is not always easy on clinical and hematological ground. The true value of this method of investigation has been, however, invalidated by the recent observation of cases of T cell ALL and by the possible existence of B cell ALL's [3, 4, 10–13, 15]. Moreover, some considerations come out from the evaluation of the rate of fluorescent cells in peripheral blood of our patient during a complete marrow remission. The demonstration of a higher than usual quantity of B lymphocytes stands for a possible monoclonal origin of at least some morphologically normal cells. The study of surface markers thus seems to us a new substantial parameter for the evaluation of the situation of rest or activity of the disease.

References

1 AISENBERG, A. C. and BLOCH, K. J.: Immunoglobulins on the surface of neoplastic lymphocytes. New Engl. J. Med. *287*: 271 (1972).

2 ASTALDI, A., jr., MORI, P. G., MARTINI, A., GIOVANELLI, A., FRANCHINI, M. L., and MASSIMO, L.: Markers and properties of a pure cell population in acute lymphocytic leukemia. Boll. Ist. siero. ter. milan. *52*: 4–6 (1973).

3 BORELLA, L. and SEN, L.: T cell surface markers on lymphoblast from acute lymphocytic leukemia. J. Immun. *111*: 1257–1260 (1973).

4 BROWN, G., GREAVES, M. F., LISTER, T. A., RAPSON, N., and PAPAMICHAIL, M.: Expression of human T and B lymphocyte cell surface markers on leukaemic cells. Lancet *ii*: 753–755 (1974).

5 CASTLEBERRY, R. P., TOBEN, H. R., and MORENO, H.: An evaluation of malignant cell origin in lymphosarcoma of childhood. Abstract. Blood *43*: 1025 (1973).

6 GAJL PECZALSKA, K. J., HANSEN, J. A., BLOOMFIELD, C. D., and GOOD, R. A.: B lymphocytes in untreated patients with malignant lymphoma and Hodgkin's disease. J. clin. Invest. *52*: 3064–3073 (1973).

7 GAJL PECZALSKA, K. J., KERSEY, J. H., NESBIT, M. E., and GOOD, R. A.: B cell proliferation manifested as acute lymphosarcoma cell leukemia and lymphoma in adolescents. Abstract. Pediat. Res. *7*: 349 (1973).

8 GOVERNA, M., MASSIMO, L., ROSANDA, C., FRANCHINI, M. L., and TONDA, G. P.: An attempt to characterize lymphocytes of acute lymphoid leukemia as T or B cells. Biomedicine *19*: 354–357 (1973).

9 KAPLAN, J, MASTRANGELO, R., and PETERSON, W D jr Childhood lymphoblastic lymphoma a cancer of thymus-derived lymphocytes Cancer Res 34 521 525 (1974)
10 KERSEY, J H, GAIL-PECZALSKA, K J, and NESBIT, M E The lymphoid system abnormalities in immunodeficiency and malignancy J Pediat 84 789–796 (1974)
11 KERSEY, J H, NESBIT, M E, LUCKASEN, J, HALLGREN, H, SABAD, A, JUNIS, E, and GAIL PECZALSKA, K J Acute lymphoblastic leukemic and lymphoma cells with thymus-derived (T) markers, in Sézary s syndrome symposium, New York 1974 (Academic Press in press)
12 KERSEY, J H, SABAD A GAIL PECZALSKA, K J, HALLGREN, H, JUNIS E, and NESBIT, M E Acute lymphoblastic leukemic cells with T (thymus-derived) lymphocyte markers Science, N Y 182 1355–1356 (1973)
13 MATHÉ G, BELPOMME, D, DANTCHEV, D, POUILLART, P, JASMIN, C, MISSET, J L, MUSSET, M, AMIEL, J L SCHLUMBERGER, J R, SCHWARZENBERG, L, HAYAT, M, VASSAL, F DE and LAFLEUR M Immunoblastic acute lymphoid leukaemia Biomedicine 20 334–340 (1974)
14 MELIEF, C J M SCHWEITZER M, ZEYLEMAKER, W P, VERHAGEN, E H and EIJSVOOGEL, V P Some immunological properties of lymphoid cells from patients with acute lymphatic leukaemia (ALL) Clin exp Immunol 14 1–13 (1973)
15 SELIGMANN, M, PREUD HOMME J L, and BROUET, J C B and T cell markers in human proliferative blood diseases and primary immunodeficiencies, with special reference to membrane-bound immunoglobulins. Transplantn Rev 16 85–113 (1973)
16 SMITH J L BARKER C R, CLEIN G P, and COLLINS, R D Characterization of malignant mediastinal lymphoid neoplasm (Sternberg sarcoma) as thymic in origin Lancet I 74–77 (1973)
17 STEIN H LENNERT, K, and PARWARESCH M R Malignant lymphomas of B cell type Lancet II 855–857 (1972)
18 WILSON, J D and NOSSAL, J V Identification of human T and B lymphocytes in normal peripheral blood and in chronic lymphocyte leukaemia Lancet ii 788–791 (1971)

PAOLO MOSCATELLI Chair of Human Genetics of the University and Pediatric Department of the Galliera Hospital Via Volta 8, I 16128 Genoa (Italy)

Acta haemat. 55 174–180 (1976)

The Action of Seed and Other Reagents on HEMPAS Erythrocytes

G. W. G. Bird and June Wingham

Regional Blood Transfusion Service, Birmingham

Key Words Blood groups · Congenital dyserythropoietic anaemia · HEMPAS · Hereditary anaemias · Lectins · Seed agglutinins

Abstract Erythrocytes of a patient with hereditary erythroblastic multinuclearity with a positive acidified serum test (HEMPAS) were tested with many seed extracts and various other reagents. Serological evidence of membrane abnormality was confirmed. Various anti-H reagents reacted relatively poorly with HEMPAS cells. HEMPAS cells have both enhanced i and depressed H antigens. A brief note on a 'new' anti-H lectin (*Cytisus glabrescens*) is provided.

Hereditary erythroblastic multinuclearity with a positive acidified serum test (HEMPAS), or congenital dyserythropoietic anaemia, type II, is an inherited (autosomal recessive) anaemia in which there is a structural abnormality of the red cell membrane [6, 7, 14] which may be responsible for defective cell division, failure of the multinucleated erythroblasts to expel their nuclei and for characteristic serological reactions. HEMPAS red cells are agglutinated at 20 °C by about one in three normal (acidified) sera, which contain a naturally occurring IgM alloantibody, anti-HEMPAS, which can be specifically absorbed by HEMPAS cells. In the presence of complement, this antibody lyses HEMPAS cells. The antibody is not present in the sera of HEMPAS patients. The amount of lysis depends on the strength of the antibody, the temperature of incubation and the serum-to-cell ratio [6]. Because HEMPAS red cells are agglutinated by many human sera, irrespective of blood group, they may be classified as polyagglutinable. The i-antigen strength of HEMPAS is greatly enhanced and may exceed the i antigen strength of newborn infants. The strength of the I antigen may be either within normal limits or increased, depending on the source of anti-I, and HEMPAS cells are readily prone to lysis by anti-I and anti-i in the presence of complement.

Because seed agglutinins have been usefully applied to the study of various red cell membrane anomalies and to the elucidation of various forms of erythrocyte polyagglutination [1, 4], we have studied the action of seed and other reagents on HEMPAS erythrocytes

Materials and Methods

Seed extracts were prepared and tested by standard methods [2–4] HEMPAS erythrocytes were obtained through the kindness of Mrs. MARIE CROOKSTON of Toronto (4 samples) and Dr A. GERBAL of Paris (3 samples) All tests were carried out on the cells of M F (group O) the patient described by CROOKSTON *et al* [7] The other HEMPAS cells were used to confirm apparently significant observations made on the cells of M F Appropriate normal control cells were tested throughout. Preliminary tests for avidity (speed of reaction by the tile method) were done first, and titre scores by the tube method were determined whenever there were significant differences in avidity In our experience, avidity of agglutination on a tile often gives a sharper indication of seed agglutinin specificity than titres or titre scores obtained in tube tests

Results

Tests on M F were carried out in 1973 and the confirmatory tests on other HEMPAS cells in 1974 and 1975 The results are recorded in tables I–V Since one of the blood group-specific seed agglutinins usefully applied in this study has not been previously reported, we have included a brief description of it at the end of this paper The important observations on HEMPAS erythrocytes were as follows (1) no specific seed anti-HEMPAS reagent was found, (2) the greatly enhanced reactions of HEMPAS cells with anti i sera were confirmed, (3) the I antigen strength of our patient was within normal limits, (4) the H antigen as measured by several anti H reagents was clearly shown to be depressed This was particularly noticeable with the anti H agglutinins from *Cytisus sessilifolius* seeds and from the eel (*Anguilla anguilla*) which react much less avidly and have lower titer scores with HEMPAS than with normal cells, (5) some 'non specific seed reagents, e g *Maclura aurantiaca* and *Wistaria sinensis* reacted more strongly and had higher titre scores with HEMPAS than with control cells and (6) the agglutinin from the albumin gland of the snail, *Helix pomatia* weakly agglutinated group O HEMPAS cells, but not control cells

Table I Reagents which acted with about equal avidity with HEMPAS and control cells

Aggregators	polybrene, Alcian blue, ruthenium red, lanthanum nitrate, protamine, polylysine, DEAE-dextran, methylcellulose
Lectins/protectins	*Amaranthus caudatus, Amaranthus hypochondriacus, Cytisus glabrescens*[1], *Datura fastuosa, Datura metelloides, Erythrina indica, Helix (Cepea) hortensis, Lens esculenta, Sophora japonica, Solanum tuberosum, Tamarix gallica, Viscum album*
Human sera	anti I

[1] Had lower titre score however (table V)

Table II Reagents with greater avidity for HEMPAS than with control cells

'Non specific' lectins	*Clerodendron trichotomum, Erythrina christa-galli, Lathyrus tingitanus, Maclura aurantiaca, Orobus vernus, Phaseolus caracalla, Phaseolus multiflorus Pisum sativum, Quercus robur, Ricinis communis, Robinia viscosa, Sambucus nigra, Triticum vulgare, Wistaria sinensis*
Human sera	anti i
Specific lectins/protectins	*Bauhinia variegata* (anti N) (M F is group NN), *Cerastium tomentosum* (anti H)[1], *Evonymus europaeus* (anti B+H)[1], *Helix pomatia*[2] (anti A cross reacts with neuraminidase-treated B or O cells), *Moluccella laevis* (anti A+N) (M F is group NN), *Petteria ramentacea* (incomplete anti H), *Ulex europaeus* (anti H inhibited by L fucose)[1]

[1] Had lower titre scores however (table V)
[2] Agglutinated M F cells weakly, no reaction with control O cells

Table III Reagents which reacted less avidly with HEMPAS than with control cells (anti H)

Anguilla anguilla[1], *Cytisus sessilifolius*[1], *Laburnum alpinum, Lotus tetragonolobus*

[1] Appreciably poorer avidity

Discussion

The remarkable enhancement of the i antigen in the presence of a normal amount of I, which was confirmed in our studies, contradicts the belief once held that there is a reciprocal relationship between I and i This contradiction is not confined to HEMPAS, it is also seen in other anaemias associated with bone marrow stress, e g thalassaemia major [10]

Table IV Reagents negative with both HEMPAS and controls, i e no HEMPAS-specific reagent found

---

Lectins *Acacia dealbata*, *Adonis aestivalis*, *Agastache mexicana*, *Akocanthera spectabilis*, *Anchusa capensis*, *Angelica archangelica*, *Anthyllis montana rubra*, *Asclepias curassavica*, *Asparagus meyersii*, *Aucuba japonica*, *Basella rubra*, *Beta vulgaris*, *Bignonia tweediana*, *Caccalia cochinea*, *Calpurnia aurea* (anti-A + B), *Cardiospermum halicacabum*, *Carica papaya*, *Catalpa bignonioides*, *Catananche coerulea*, *Ceanothus americana*, *Cladrastis tinctoria*, *Clarkia elegans*, *Coleus candida*, *Cordyline indivisa*, *Corokia cotoneaster*, *Coronilla emerus*, *Cotyledon simplicifolia*, *Cuphea lanceolata*, *Cyphomandra betacea*, *Cytisus maderensis*, *Cytisus nigricans*, *Cytisus supinus*, *Datura suaveolens*, *Dicentra spectabilis*, *Didiscus coerulea*, *Digitalis mertonensis*, *Dolichos biflorus* (anti-A_1), *Echium plantaginium*, *Echium rubrum*, *Elscholtzia stauntonii*, *Eruca sativa*, *Euphorbia lathyrus*, *Fatsia japonica*, *Gaura lindheimerii*, *Gingko biloba*, *Gleditschia triacanthus*, *Glycine soja*, *Griselinia littoralis*, *Gypsophila pacifica*, *Helianthus annuum*, *Holboellia latifolia*, *Holmskildia sanguinia*, *Horminum pyrenaicum*, *Hovenia dulcis*, *Humulus japonicus variegatus*, *Hyssopus aristatus*, *Iberis coronaria hyacinthifolia*, *Iberis jucunda*, *Iberis sempervirens*, *Iberis umbellata*, *Ionopsidium acaule*, *Isatis tinctoria*, *Kaulfussia amelloides*, *Lagenaria leucantha*, *Lavendula spica*, *Layia elegans*, *Leonotis leonuris*, *Leontopodium alpinum*, *Leschenaultia biloba*, *Lespedeza sieboldii*, *Leucospermum bolusii*, *Liatris pycnostachya*, *Limnanthus douglasii*, *Linum alpinum*, *Linum utilitissimum*, *Lonicera caprifolium*, *Lotus corniculata*, *Luffa*, *Lupinus speciosa*, *Lychnis coeli-rosa*, *Martynia louisiana*, *Medicago echinus*, *Melia azederach*, *Monarda didyma*, *Nepeta macrantha*, *Nicandra physaloides*, *Ocimum minimum*, *Omphalodes linifolia*, *Papaver somniferens*, *Paronychia capitata*, *Perilla frutescens*, *Phacelia campanularia*, *Philodendron bipinnatifidum*, *Phlomis samia* (anti-A + B), *Phlomis viscosa* (anti A + B), *Phytolacca americana*, *Picnostachys urticifolia*, *Pueraria lobata*, *Quamoclit lobata*, *Rumex scutatus*, [illegible] (anti-Tn), *Salvia splendens*, *Sanguinia canadensis*, *Saponaria calabrica*, *Sarcococca con-* [illegible] *Thymus serpyllum*, *Torenia fournieri grandiflora*, *Verbascum bombyciferum*, *Viscaria candida*, *Zea mays*

Human sera 'incomplete' Rhesus antibodies e g. anti-D with HEMPAS D + ve cells in saline

---

Table V Titre scores with various reagents

	HEMPAS	Control
Anti H reagents		
Anguilla anguilla (anti H)	16	38
Cerastium tomentosum (weak extract)	5	10
Cytisus glabrescens	23	44
Cytisus sessilifolius	25	47
Evonymus europaeus (anti B+H)	44	47
Human anti H (Bombay plasma)	13	21
Laburnum alpinum	not tested, extract too weak	
Lotus tetragonolobus (weak extract)	7	13
Ulex europaeus	32	51
Miscellaneous		
Maclura aurantiaca (syn *pomifera*)	92	77
Wistaria sinensis	46	16

and malignant disorders of lymphoreticular tissues [11] An explanation must await the full chemical characterization of I and i and elucidation of their biosynthesis

A relationship between I, i and H is already known [2] Our observations show that in HEMPAS cells there is a reciprocal relationship between H and i ROCHANT *et al* [12], independently tested the red cells of a HEMPAS patient with an extract of *Ulex europaeus* seeds, with negative results, but observed that anti-H was not present in the patient's serum We do not know whether the H antigen of their patient's red cells was very much more depressed than those of our patients, or whether their *Ulex* extract was very weak We confirmed the weak agglutination of HEMPAS cells by the *Helix pomatia* protectin reported by BIZOT and MONIS [5] The *Helix hortensis* protectin, however, reacted about equally with HEMPAS and control cells

Early in our investigation, we confirmed that HEMPAS cells were different from other known forms of polyagglutinable erythrocytes T, Tk, Tn, which are acquired characters, and Cad (Cad 1) which is an inherited form of polyagglutination The non-identity of HEMPAS with any of these forms of polyagglutination was demonstrated by cross-absorption studies and by the reactions of various seed reagents For example, HEMPAS cells are not agglutinated by the lectin of *Arachis hypogaea* (anti-T)

or *Salvia sclarea* (anti-Tn), *Salvia horminum* (anti-Tn + anti-Cad) or *Dolichos biflorus* (anti-A_1, anti-Tn and anti Cad) [1, 4] CROOKSTON and CROOKSTON [8] have shown that HEMPAS is different to T, BIZOT and MONIS [5] that it is different to Cad, and ROCHANT *et al* [12] that it is not T, Tn or Cad

The En(a–) character, in which there is a red cell membrane defect, was also distinguished from HEMPAS En(a–) cells are not polyagglutinable They are agglutinated in saline by 'incomplete' Rhesus antibodies and by the lectin of *Sophora japonica* [9] We found that HEMPAS cells (M F) were not agglutinated either by 'incomplete' Rhesus antibodies in saline or by the *Sophora japonica* lectin which reacts strongly with En(a–) cells or those of En(a+) heterozygotes, this was first observed by SANGER [13] who also observed that these cells in saline do not react with incomplete anti D, unlike En(a–) or En(a+) heterozygous cells, that they reacted normally with rabbit or human anti En^a and reacted more strongly than a normal NN control with *Vicia graminea* and *Bauhinia purpurea* lectins Furthermore, the *Maclura aurantiaca* lectin, which reacts relatively poorly with En(a–) cells [3], reacts more strongly with HEMPAS cells (table V)

Brief note on a 'new' anti-H lectin Because of the difficulty sometimes experienced in obtaining seeds of various plants for the preparation of blood grouping reagents, it is always useful to learn of new ones In March 1973, during one of our periodic searches for 'new' lectins, we found an anti H agglutinin in the seeds of *Cytisus glabrescens* Extracts of the seeds reacted on red cells in the following descending order of avidity O, A_2, B, A_1 O_h cells were not agglutinated Titre scores were as under O = 56, A_2 = 52, B = 25, A_1 = 10 The avidity and titration results are typical of anti H [2] The agglutinin was inhibited by secretor, but not non secretor, saliva L-fucose did not inhibit, *N*-acetylglucosamine inhibited only slightly, but salicin neutralized the lectin (titre 32) Thus the *Cytisus glabrescens* anti H agglutinin is of the *Cytisus sessilifolius* type, not the *Lotus tetragonolobus* eel serum type of anti H, which is neutralized by L-fucose It is quite a good anti H and would be suitable for routine laboratory use The *Cytisus glabrescens* agglutinin, as did other anti H reagents reacted relatively poorly with HEMPAS cells (table V)

Acknowledgements We are grateful to Mrs. MARIE CROOKSTON for sending us HEMPAS bloods, for expert advice and much valuable information and to Dr A. GERBAL for providing blood samples from HEMPAS patients.

References

1 BIRD, G W G Erythrocyte polyagglutination Nouv Revue fr Hémat *11* 885–896 (1971)

2 BIRD, G W G and WINGHAM, J Anti H from *Cerastium tomentosum* seeds A comparison with other seed anti H agglutinins Vox Sang *19* 132–139 (1970)

3 BIRD, G W G and WINGHAM, J The action of seed and other reagents on En(a–) erythrocytes Vox Sang *24* 48–57 (1973)

4 BIRD, G W G and WINGHAM, J Haemagglutinins from *Salvia* Vox Sang *26* 163–166 (1974)

5 BIZOT, M et MONIS, M Un cas de dysérythropoïèse congénitale de type II Revue fr Trans *17* 147–150 (1974)

6 CROOKSTON, J H, CROOKSTON, M C, BURNIE, K L, FRANCOMBE, W H, DACIE, J V, DAVIS, J A, and LEWIS, S M Hereditary erythroblastic multinuclearity with a positive acidified serum test a type of congenital dyserythropoietic anaemia Br J Haemat *17* 11–26 (1969)

7 CROOKSTON, J H, CROOKSTON, M C, and ROSSE, W F Red cell abnormalities in HEMPAS (hereditary erythroblastic multinuclearity with a positive acidified serum test) Br J Haemat *23* 83–91 (1972)

8 CROOKSTON, J H and CROOKSTON, M C Hereditary anemia with multinuclear erythroblasts (HEMPAS) Birth Defects *8* 15–19 (1972)

9 FURUHJELM U, MYLLYLA, G, NEVANLINNA, H R, NORDLING, S, PIRKOLA, A, GAVIN, J, GOOCH, A, SANGER, R, and TIPPETT, P The red cell phenotype En(a–) and anti Ena serological and physicochemical aspects Vox Sang *17* 256–278 (1969)

10 GIBLETT, E R and CROOKSTON, M C Agglutinability of red cells by anti i in patients with thalassaemia major and other haematological disorders Nature, Lond *201* 1138–1139 (1966)

11 ROCHANT, H, TON THAT, H, N GO MINH, M, LETAOU, J, HENRI A et DREYFUS B Etude quantitative des antigènes érythrocytaires I et i en pathologie Nouv Revue fr Hémat *13* 307–318 (1973)

12 ROCHANT, H, N GO MINH, M, TON THAT, H, HENRI, A, BASCH, A, SULTAN, C et DREYFUS, B Un nouveau cas de dysérythropoïèse congénitale de type II Caracteres immunologiques et transmission héréditaire de l antigène de polyagglutinabilité distinct des antigènes T, Tn et Cad Nouv Revue fr Hémat *13* 649–660 (1973)

13 SANGER, R Personal communication to M C CROOKSTON (1969)

14 VERWILGHEN, R L, LEWIS, S M, DACIE, J V, CROOKSTON, J H and CROOKSTON, M C HEMPAS congenital dyserythropoietic anaemia (type II) Q Jl Med *42* 257–278 (1973)

Dr G W G BIRD, Regional Blood Transfusion Service, *Birmingham, B15 2SG* (England)

Acta haemat. 55 181–191 (1976)

Combined Hereditary Deficiency of Factors VII and VIII

A Distinct Coagulation Disorder Due to the 'Lack' of an Autosomal Gene Controlling Factor VII and VIII Activation?[1]

A. GIROLAMI, R. VENTURELLI, G. CELLA, L. VIRGOLINI and A. BURUL

University of Padua Medical School, Institute of Semeiotica Medica (Director: Prof. M. AUSTONI), Padua

Key Words. Blood coagulation · Coagulation disorders · Factor VII and VIII deficiency

Abstract. A patient with a combined hereditary deficiency of factors VII and VIII is presented together with a family study. The main bleeding manifestations were easy bruising and bleeding after tooth extractions. No hemarthrosis was ever observed. The main laboratory features consisted in a mild prolongation of prothrombin time and of partial thromboplastin time. TG test was abnormal and was corrected by the addition of adsorbed normal plasma. Specific assays revealed a moderate defect of factors VII and VIII. All other clotting factors were within normal limits. The factor VII antigen in the propositus was normal or nearly normal. The factor VIII-associated antigen was also normal. Five additional family members presented the same coagulation pattern and were variably symptomatic. The hereditary transmission pattern seems to be autosomal dominant. The defect appears to be due to a structural abnormality of a gene controlling factors VII and VIII activation.

Hereditary deficiencies of more than one clotting factor are very rare coagulation disorders. The only condition which seems to be an indipendent nosological entity is combined deficiency of factors V and VIII. About 20 patients with such a peculiar defect have already been satisfactorily documented [9, 12, 17, 24–27]. Similar considerations could be formulated for combined deficiencies of the prothrombin complex factors [4, 23]. But the matter is still disputed particularly because only 2 cases have been described and because the defect seemed to be correctable almost completely by parenteral administration of vitamin K [4, 23]. Therefore, the possibility of an acquired defect secondary to impaired vitamin K adsorption remains

[1] This study was supported in part by a Grant from the CNR (grant 74.00189.04).